

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :
Prasad DEVARAJAN et al. : Confirmation No: 2792
Serial No.: 10/811,130 : Group Art Unit: 1641
Filed: March 26, 2004 : Examiner: FOSTER, Christine E.
A METHOD AND KIT FOR DETECTING THE EARLY
ONSET OF RENAL TUBULAR CELL INJURY

DECLARATION UNDER 37 CFR 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

We, Jonathan M. Barasch and Prasad Devarajan, do hereby declare as follows:

1. We are the joint inventors of the subject matter described and claimed in the above-identified patent application.

2. I, Jonathan M. Barasch, hold an undergraduate degree in Biochemistry from Dartmouth College and a PhD and Medical Doctor (M.D.) degrees from the College of Physicians and Surgeons. I also completed a residency in Internal Medicine and a fellowship in Clinical Nephrology at Columbia-Presbyterian Medical Center ("Columbia-Presbyterian") in New York. I have been conducting research in nephrology since 1990. I am currently Associate Professor of Medicine and Cell Biology at Columbia University ("Columbia"), and an Assistant Attending Physician in Medicine at Columbia-Presbyterian. I am also the Director of the Research Track of the House Staff Training Program at Columbia.

3. I, Prasad Devarajan, hold an undergraduate degree in Biology and a Medical Doctor (M.D.) degree from Bombay University, India. I also completed graduate research in renal disease in the Department of Physiology, and a residency in Pediatrics at SUNY at Stony

Brook, NY. I also completed a fellowship in Nephrology at Yale University, and completed an NIH-sponsored research fellowship in renal disease at Yale. I have been conducting research in the field of renal disease since 1985. I am presently the Louise M. Williams Endowed Chair, Professor of Pediatrics, Professor of Developmental Biology, Director of Nephrology and Hypertension, Director of Nephrology Clinical Laboratories, CEO of Dialysis Unit, at Cincinnati Children's Hospital Medical Center and the University of Cincinnati School of Medicine, Cincinnati, OH.

I am an expert reviewer of grant applications in the field of renal diseases for the NIH and several other national and international organizations. I am an expert reviewer of publications submitted to more than 20 scientific and medical journals in the field of renal disease. I am a member of the Editorial Board of key journals in the field of renal disease. I am on the Advisory Board and Research Committees of the American Society of Nephrology, American Society of Pediatric Nephrology, International Acute Kidney Injury Network, and the National Institutes of Health in the field of renal disease.

4. We have read and are familiar with the presently-amended claims of the above-identified patent application, with the Office Action mailed June 9, 2009, and with patent and journal article references recited herein.

5. The Office Action recites (page 7 lines 3-9) that Matthaeus 1 teach that levels of NGAL protein are upregulated in response to experimentally induced acute ischemic renal injury in a rat model (i.e., ischemic renal tubular cell injury; see entire selection). By contrast, control animals displayed only minor expression of NGAL, demonstrating that renal injury and repair is associated with an upregulation of NGAL (i.e., correlating the level of NGAL with ischemic renal tubular cell injury). Matthaeus 1 further state that NGAL was elevated ‘after 24 and 48 hours’ of renal ischemia as assessed by Western blot analysis” in kidney tissue.

6. The Office Action continues on (page 7 lines 20 to page 8 line 4) to state that the “Matthaeus references make clear that their studies were performed on rats *as an animal model of human disease* (this is made explicit in Matthaeus 2, who refer to a ‘rat model of renal

ischemia’). Matthaeus 1 state that the purpose of their experiment is to ‘further elucidate the processes involved in renal injury and repair’. The findings reported therein support a ‘critical role in the renal response to injury’ for NGAL, correlating upregulation of NGAL with ischemic renal tubular cell injury.” (emphasis in original).

7. The Office Action (page 7 lines 14-19) also states that the Matthaeus references differ from the claimed invention in that they fail to specifically teach detecting NGAL in **urine** as claimed; rather, the references detected NGAL in the postischemic kidney (tissue)...(and) the references fail to teach detecting NGAL in urine samples taken “*within four hours of the RTCP*”, and “detected NGAL protein expression via Western blot analysis, but are silent as to whether their procedures involved formation and detection of an antibody-NGAL complex” (emphasis in original).

8. We do not agree with the Office Action’s characterization of Matthaeus (hereinafter, Matthaeus 1 and Matthaeus 2 will be referred to collectively as “Matthaeus” since their disclosures are nearly identical). First, we shall correct characterizations made in the Office Action by emphasizing that Matthaeus stated “(w)e conclude that *MMP-9 and NGAL* may play a critical role in the renal response to ischemic injury.” (emphasis added) Matthaeus repeatedly mentions the association of MMP-9 and NGAL; specifically, “NGAL has been shown to occur in disulfite-linked complexes with matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-9 (TIMP-1)”; “(e)xpression of NGAL and *its associated molecules*, MMP-9 and TIMP-1, was studied”; and “(a)s MMP-9 and NGAL are simultaneously upregulated in injured proximal tubuli...”.

9. We do not believe that Matthaeus’ emphasis on an association between MMP-9 and NGAL can be so easily trivialized by focusing only on NGAL and its role. It was thought at the time of Matthaeus that the interaction with MMP-9 was the key function of NGAL. For example, Yan et al [The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL), J Biol Chem. 2001 Oct 5;276(40):37258-65] showed that NGAL was capable of protecting MMP-9 from degradation in a dose-dependent manner and thereby preserving MMP-9 enzymatic

activity. Yan teaches that the complex was a marker of cancer, and hypothesized that NGAL might be involved in tumor progression via its interaction with MMP-9 in view of other recent findings showed that NGAL expression levels were up-regulated in colorectal neoplasia and several epithelial carcinomas.

We point out that the NGAL reported by Yan et al was not the same composition of the NGAL that is the subject of our invention. The size of the NGAL they report is 35,000 whereas the NGAL we report is 25,000.

10. Second, and importantly, the NGAL:MMP-9 is a cancer marker that results from chemical linkages. These chemical (disulfide linkages) are even more common in rodents than in humans (and Matthaeus used rodents) because in rodents there is an extra-unpaired cysteine residue in rodent NGAL (but not in the human) and this amino acid is involved in cross-linking. We would conclude that Matthaeus is focused on the biology of MMP-9 activity in the body of the kidney, prominent in rodents.

11. The MMP-9/NGAL complex was known to be covalently associated, and hence its separation into components would be understood to require chemical reactants. Matthaeus' description would suggest that the NGAL in the proximal tubuli was associated with MMP-9 in the kidney tissue as the MMP-9/NGAL complex, which is not the same chemical compound as NGAL alone. Indeed, the function of NGAL in Matthaeus relates to its association with MMP-9 and its proteolytic activity.

12. The Office Action asserts that Matthaeus teaches *correlating* upregulation of NGAL with ischemic renal tubular cell injury. Quite the contrary, Matthaeus teach that levels of NGAL *and* MMP-9 protein are upregulated in response to experimentally induced acute ischemic renal injury in a rat model, and that renal injury and repair was associated with an upregulation of NGAL *and* MMP-9. However, the brief description of Matthaeus suggests only a single datum point of NGAL protein detection in the kidney tissue (not in urine) that is made long after the initial induced injury. From this single datum, we could not conclude that the upregulation of NGAL teaches "correlating the level of NGAL with ischemic renal tubular cell injury", as the Office Action alleges. A correlation requires a determination of the strength and direction of a

relationship between two variables. Matthaeus' limited results fail to establish any such alleged correlation between NGAL and ischemic renal tubular cell injury.

13. The rejection's actual characterization of Matthaeus' teaching fails to mention, and therefore ignores the importance of, the location and timing of NGAL and MMP-9 expression. As will be discussed below in item 29, the actual location and timing of NGAL expression described in Matthaeus is important and cannot be ignored by any appropriate characterization of the teaching on Matthaeus, notwithstanding that these are also important differences between the disclosure of Matthaeus and the claimed invention.

14. We also note that Matthaeus do not describe the technique used for inducing an ischemic renal injury, other than a "rat model of renal ischemia" and the "postischemic kidney". Matthaeus does not describe whether only one kidney (unilateral ischemia) or both kidneys (bilateral ischemia) were clamped, or for how long. In other references authored by Bonventre (the last named author of Matthaeus), "postischemic kidney" refers exclusively to a unilaterally-clamped kidney (see Ichimura et al, The Journal of Biological Chemistry, Vol. 273, No. 7, pp. 4135-4142, February 13, 1998) and US Patent 6,664,385 (Sanicola-nadel et al). As described in our specification (paragraph [0083]), mice with unilateral renal ischemia display serum creatinine levels that are indistinguishable from control animals, while mice with bilateral ischemia show significant elevation of serum creatinine. Therefore, there would be considerable uncertainty as to the extent of ischemic renal injury induced on the rat subjects of Matthaeus, and no predictability whether such rat subjects would have exhibited elevated levels of serum creatinine that accompany acute renal failure (ARF).

15. The Office Action also states (page 8 lines 5-8) that "(i)t was well known in the art that disease processes may produce changes in the levels of certain specific analytes, and that measurement of the levels of such analytes can be used to detect the presence of the disease. This is taken to be admitted prior art because applicant has failed to traverse this assertion (according to MPEP 2144.03)."

16. We do not agree with the Office Action's characterization. The assertion fails because the alleged "common knowledge statement" is at best a generalized principle that is devoid of specifics. The statement "disease processes generally may produce changes in the levels of certain specific analytes, and that measurement of the levels of such analytes can be used to detect the presence of the disease" lacks the specifics for any person to either support or refute the statement, and was understood to be a generalized concept that may or may not be true depending on the specifics, which are completely lacking. That is, the alleged statement may or may not be true, and unpredictably so, depending on the specific disease, the specific analyte, and the timing and degree of change in the level of specific analyte. The statement establishes no predictability that NGAL can be associated with an ischemic renal injury outside of the context described in Matthaeus: that is, within kidney tissue, and specifically only in the proximal tubuli, after 24 hours. Any alleged failure to have refuted such statement does not extend the scope of the statement to any specific disease or analyte in any way.

17. The Office Action goes on to state, at page 8 lines 9-13, that "it would have been obvious to one of ordinary skill in the art to detect NGAL for the purpose of diagnosing acute renal injury in light of the teachings of Matthaeus that NGAL is specifically elevated in this disease condition, and further in view of the general knowledge of one skilled in the art that markers changed in response to disease can be used as biomarkers for diagnosis of the disease."

18. We do not agree with this characterization of Matthaeus. Matthaeus mentions nothing whatsoever about a biomarker. Based solely on the teaching in Matthaeus that NGAL associates with the protein MMP-9 in the post-ischemic kidney tissue, the examiner concludes that NGAL is a biomarker for the diagnosis of ischemic renal injury in humans. However, such a conclusion, at a very minimum, requires that the teaching of Matthaeus would lead a person of ordinary skill to recognize that NGAL has the properties and attributes of a biomarker, which in fact is completely impossible in view of Matthaeus' teaching of the expression of NGAL at the wrong place (proximal tubuli), wrong time (after 24 hours) and in the wrong form (in an association with MMP-9).

19. We note that the examiner previously has asserted the requirements for a biomarker. In the Office Action dated May 29, 2007, the examiner recognized the “laborious and lengthy nature of biomarker validation recognized by those skilled in the art, citing as examples, Bast et al. ("Translational Crossroads for Biomarkers" Clin Cancer Res 2005; 11(17),6103-6108) -- a reference that published two years *after* the filing date of this application -- , which points to the "lengthy process" of assay development and validation and notes that many markers that correlate with disease statistically may not prove to be useful clinically (p. 6105, right column). See also LaBaer et al. ("So, You Want to Look for Biomarkers" Journal of Proteome Research 2005; 4, 1053-1059), which teaches that crucial validation steps are needed to demonstrate that an identified biomarker is a reliable predictor, and also that the process of converting such a biomarker into a practical clinical test is even more daunting (p. 1053, see the paragraph bridging the left and right columns). Baker ("In Biomarkers We Trust?" Nature Biotechnology 2005; 23(3),297-304) also speaks to the unpredictability involved in clinically applying biomarkers (see p. 298, the section "Walking on Thin Ice"): "Using a new biomarker is like walking across a frozen lake without knowing how thick the ice is," says Ole Vesterqvist ... "You start walking, and you get comfortable. Then you break through." The examiner concluded that “the state of the art teaches the unpredictability associated with the clinical use of biomarkers even after a biomarker has been correlated with a specific disease state”.

20. A successful or “ideal” diagnostic biomarker would be characterized by three features: a) its expression would be tightly correlated with the disease; b) it would be easily (non-invasively) detectable, and c) it would allow detection at the earliest stage possible. The question to be addressed is therefore whether the skilled person, with Matthaeus in hand, **would** have identified NGAL as a biomarker of renal ischemia. However, in fact, the kidney NGAL described by Matthaeus is present at i) the wrong place, ii) at the wrong time, and iii) in the wrong chemical configuration.

21. Feature a) of a diagnostic biomarker requires that the expression of the biomarker in the sampled medium (e.g., urine) be tightly correlated with the disease, in this case ischemic renal injury, including an ischemic renal injury that can progress to acute renal failure. Matthaeus identify that NGAL protein is associated with MMP-9 in the injured kidney tissue,

and is upregulated in the injured proximal renal tubuli of the ischemic kidney, but do not mention or suggest the urine.

22. Consequently, Matthaeus teaches nothing about a correlation of the expression of urinary NGAL with ischemic renal injury, let alone “a tightly correlated expression” with a renal tubular cell injury.

23. Even further, as earlier mentioned in item 12, Matthaeus provide a single datum on the expression of NGAL, in the proximal tubules, at a time 24 hours after the induced renal injury. A correlation requires a determination of the strength and direction of a relationship between two variables. Consequently, the limited data provided by the Matthaeus reference(s) fail objectively to establish a “tight correlation” of the expression of NGAL with the ischemic renal injury.

24. In our opinion, the authors of Matthaeus had no expectation of NGAL as a urinary biomarker of ischemic renal injury, and a person of ordinary skill in the art reading Matthaeus would not have considered, and could not have predicted, that NGAL would be a urinary biomarker of ischemic renal injury at the time of the present invention.

25. The Office Action goes on to state (pg 8 lines 14-17 through page 9 line 13) that “it would have been further obvious to employ urine as the sample source, rather than the kidney tissue samples examined in the rat models of Matthaeus 1 and 2, for the following reasons... (that) (o)ne skilled in the art would immediately recognize that isolation of kidney tissue would be very invasive and therefore unsuitable method for diagnosing renal injury in humans,” and that “in light of the general knowledge of one skilled in the art that urine is an easily collected and non-invasive sample source for assay of biological analytes (as taught for example by Ramsden et al.), it would have been obvious to use urine as the sample source instead of kidney tissue samples when detecting NGAL for diagnosis of ischemic renal injury in human subjects, for the advantage of being a non-invasive and easily collected sample.”

26. We do not agree with this characterization of Ramsden et al. Ramsden relates to detection of the marijuana-metabolite THC as a drug marker in urine, and makes no mention or suggestion of biomarkers or renal injury or disease. The alleged teaching of Ramsden et al that urine samples are noninvasive and convenient is so general that it lacks any teaching whatsoever.

27. We have pointed out in previous responses in the examination of this application that a person of ordinary skill would have found that isolating kidney tissue (either excising portions or homogenizing the whole kidney) was entirely necessary and appropriate for the purposes of study that was the subject of Matthaeus, which was to observe the pattern and location of mRNA and protein expression in a post-ischemic kidney. The invasiveness of a method depends on the context of its use. For the purpose identified in Matthaeus of studying the synthesis and co-expression of NGAL, MMP-9 and TIMP-1, a person of ordinary skill would find the invasive isolation of kidney tissue entirely appropriate (see our remarks in the Response of June 3, 2008, pages 15-16). The stated findings of Matthaeus 1 and 2, that NGAL protein expression was upregulated after 24 and 48 hours, and that immunocytochemistry of NGAL revealed bright fluorescence in the most extensively damaged areas (the "injured proximal tubuli") could not have been determined if urine sampling were substituted for the methodology of Matthaeus. Therefore, for the purposes taught in Matthaeus 1, a person of ordinary skill in the art would consider urine sampling neither a suitable nor obvious substitute for the method of isolating kidney tissue disclosed in Matthaeus.

28. Feature b) of the diagnostic biomarker requires the possibility of easy detection. This feature is not disclosed or suggested by Matthaeus. Matthaeus do not indicate an alternative "window of observation" for NGAL that would allow instead for its non-invasive monitoring. When it comes to *where* the NGAL actually shows up, Matthaeus do not go beyond saying that NGAL protein expression was "*upregulated in the injured proximal tubuli.*"

29. The main function of the proximal renal tubules is reabsorption and degradation of proteins filtered from the circulating blood stream. The presence of NGAL protein in the proximal tubuli would indicate the capture of NGAL from the glomerular filtrate which is derived not from the kidney itself but from the circulating serum. Most substances that appear in

the proximal tubules of the kidney do not necessarily then also show up in the urine. This is also true for NGAL. Matthaeus in fact confirms this, by finding that NGAL was accumulated in the proximal tubuli, where the megalin receptors had captured and were in the process of degrading (via lysosomes) the NGAL protein, as is the normal process. This would have then informed a person of ordinary skill in the art that the then well-known megalin process was functional. If so, a person of ordinary skill in the art would not have predicted that NGAL that was observed in the proximal tubuli at 24 hours would have been likewise observed in urine. The capture of NGAL by the proximal tubuli is likely to be exclusive of any potential urinary location.

30. It is well known that proteins absorbed in the proximal tubules exclusively are degraded via the lysosomal pathway, and they thus do not pass into the urine. For example, the "The Kidney" textbook by Brenner and Rector, third edition of 1986 and its page 30, (Appendix B) gives a good description, starting on the left side, first full paragraph, of the reabsorption and degradation of plasma macromolecules and proteins ("proximal tubuli endocytosis"). Thus, proximal tubuli endocytosis is a well-known phenomenon and has been studied in detail by various researchers, including others at the time of the invention.

31. Christensen in *Am. J. Physiol. Renal Physiol.* 280: F562-F573, 2001 (Appendix C) has investigated the role of *inter alia* megalin which is one of the receptors involved in proximal tubuli endocytosis (see text starting at page F565, at bottom of left column, through page F566, right column, 2nd para). Megalin mediates the endocytosis of a wide variety of ligands of considerably differing chemical nature, structure and constitution, many of which are proteins of various sizes (see in particular table 1 on F566).

32. Nykjer et al. in *Cell*, Vol.96, 507-515 (February 19, 1999) (Appendix D) reports for example in detail on the endocytosis of vitamin D-type steroids, which is only one of the many examples given in the table 1 of Christensen. Nykjer shows that endocytosis is suppressed in megalin-knock out organisms (see Nykjer, page 512 - end of the right column).

33. Christensen underscores that "(m)ost, if not all of the ligands taken up by megalin or cubilin in proximal tubuli are degraded in lysosomes" (see F655, left column) and concludes

that "Absence or dysfunction of either receptor is associated with significant proteinuria, showing that both are important for normal absorption of filtered protein (see F570, left column: Conclusion).

34. With this background, it appears that the skilled person would have also regarded NGAL susceptible to proximal tubuli endocytosis. Indeed, a paper published by Hvidberg *et al.* in the Federation of European Biochemical Societies (FEBS) Letters 579, 773-777 in 2005 (Appendix E), subsequently confirms, as a result of studies conducted by a separate group of researchers in a period of time close to the filing date of our Application, that NGAL is deemed prone to being strongly absorbed by megalin.

35. Hvidberg reports: "*The other receptor is megalin [8], a multi-ligand endocytosis receptor that is expressed on a variety of epithelia, primary such that have a high adsorptive capacity such as tubular epithelia cells of kidneys...*" (see page 773, right column, middle of the first paragraph); and "...*thus indicating that megalin is involved in mediating the cellular uptake of NGAL by the cells*"; "...*internalized NGAL is not recycled but segregated from the receptor and targeted to endosomes and lysosomes.*" (see page 775, left column, in section 5, reporting results, last but seven lines from the end of the paragraph, as well as conclusive statement of the said paragraph.); "(w)e demonstrate here that megalin acts as such as a cellular receptor for NGAL." (see page 775, right column, in section 6, reporting discussion, at about two thirds from the beginning of the first paragraph.); and, as a conclusion: "*The studies of uptake indicate that the capacity of megalin to endocytose NGAL is very high.*" (see page 776, left column, last sentence of the first paragraph. The Hvidberg reference comes mainly as a reported individual confirmation of the skilled man's understanding. This understanding existed in any case prior to Hvidberg.

36. These references provide evidence that urine would not be *self-evidently* understood as "the" observation window for the NGAL reported in Matthaeus. The skilled person was rather concerned with the high probability of NGAL endocytosis, and would have considered that a finding of NGAL protein expression (accumulation) in the proximal renal tubuli would suggest its absorption and degradation exclusively via the lysosomal pathway, and

would not suggest its appearance in the urine. Matthaeus themselves give the evidence that NGAL would not have been expected to appear in the urine, since it was only found in that place where it is reabsorbed.

37. A contrary finding that NGAL expressed in the renal proximal tubuli then passes into the urine would have been quite a surprise to a person of ordinary skill. Indeed I, Prasad Devarajan, was surprised when I first observed that NGAL was detected in the urine following an induced renal ischemia. It has only been through the present invention that it became known that NGAL stemming from renal tubular cell injury, is readily and visibly **detectable in the urine** and that NGAL expression in the urine can be **univocally correlated** with renal tubular cell injury as the underlying cause (see, for example, examples 5 or 6 of our specification).

38. Studies performed after the filing date of the present application (by I, Jonathan M. Barasch) have identified the distal nephron segments as the source of NGAL, including unexpected cell types such as the intercalated cell, but not the proximal tubuli.

39. We also point out that *if* any NGAL protein passed beyond the proximal tubules and if that urine might have been expected to have passed into the urine, the particular sampling practice employed by Matthaeus would have flushed out any urine.

40. The Office Action goes on to state (page 9 bottom to pg 10 line 2) that “(o)ne would have a reasonable expectation of success because it was known in the prior art that NGAL is excreted in urine, as taught by Blaser et al. and Moses et al (US 7153660). In particular, Blaser et al. teach detection of human neutrophil lipocalin (NGAL) in urine by sandwich ELISA (see in particular the abstract; page 139, section 2.4; and pages 142-143, sections 3.3-3.4). Moses et al. also teach that NGAL may be detected in human urine by Western Blot (Example 2 and Figure 1B). As such, in light of the teachings of Blaser et al. and Moses et al., one skilled in the art would have a reasonable expectation of success in using urine as a sample source for detection of NGAL in response to renal injury (rather than kidney tissue as taught by Matthaeus 1 and 2) since NGAL was known at the time of the instant invention to be excreted in urine.”

41. We do not agree with this characterization of Moses and Blaser, none of which is reasonably related to ischemic renal injury or other renal tubular cell injuries, and none of which identify NGAL specifically as a diagnostic biomarker of any specific disease. We note while both Moses and Blaser teach one is capable of detecting NGAL in a sample of urine, neither reference provides any support for any reasonable expectation of success that NGAL would be identified in the urine of a patient following an ischemic renal injury, if only because these references addressed work on different diseases.

42. Moses (US 7,153,660) relates to a method for diagnosing the presence of or prognosing appearance of metastatic cancer, by the identification of high molecular weight enzyme complexes comprising MMPs in bodily samples (abstract). The cancer is identified as organ-confined prostate cancer, metastatic prostate cancer, cancer found in cells of epithelial origin, mesodermal origin, endodermal origin or hematopoietic origin, and cancer selected from the group consisting of cancers of the nervous system, breast, retina, lung, skin, kidney, liver, pancreas, genito-urinary tract, and gastrointestinal tract (column 2 lines 22-29). The high molecular weight enzyme complexes comprising can also comprise a lipocalin, e.g., NGAL, and/or a TIMP, e.g., TIMP-1 (column 2 lines 35-37). The method also includes obtaining a biological sample from a subject and detecting lipocalin in the biological sample, and correlating the presence or absence of the lipocalin with the presence or absence of a tissue remodelling-associated condition, thereby facilitating the diagnosis of the subject for a tissue remodelling-associated condition (column 2 lines 48-54).

43. Most of the details of the Moses reference relate to supporting the finding that the 125 kDa MMP activity in urine samples of cancer patients was a complex of MMP-9 and NGAL (column 15 lines 37-39). Moses states that the origin of the 125 kDa MMP-9/NGAL activity in urine of cancer patients remains unclear, but that it is likely that MMP-9 and NGAL are separately executed into urine where they form the 125 kDa MMP-9/NGAL complex (column 17 lines 37-48). At column 5 lines 22-38, Moses states that “(m)any thousands of proteases occur naturally, and each may appear at different times of development and in different locations in an organism. The invention herein features enzymes of the class of the matrix metalloproteinases (MMPs, class EC 3.4.24). These enzymes, which require a divalent cation for activity, are

normally expressed early in the development of the embryo, for example, during hatching of an zygote from the zona pellucida, and again during the process of attachment of the developing embryo to the inside of the uterine wall. Enzyme activities such as N-acetylglucosaminidase (EC 3.2.1.50) appear in urine in the case of renal tubular damage, for example, due to diabetes (Carr, M. (1994) *J. Urol.* 151(2):442-445; Jones, A, et. al. (1995) *Annals. Clin. Biochem.*, 32:68-62). That these activities appear in urine as a result of renal tubular damage is *irrelevant* to the present invention as described herein.” (emphasis added)

44. Therefore, the only mention in Moses et al of a renal disease, other than kidney cancer as one of numerous metastatic cancers, is a reference to “renal tubular damage” associated the enzyme activity of N-acetylglucosaminidase in the urine, which activity is “irrelevant to the present invention” of Moses et al. This would suggest to us an *absence* of any correlation or association between the diagnosis of a metastatic cancer by detection of the MMP-9/NGAL complex in urine, and renal tubular damage, and does not predict any success in identifying NGAL in the urine of a patient following an ischemic renal injury.

45. We also note that Moses et al relates exclusively to a non-acute disease, specifically metastatic cancer, and that it is not known which latency period may expire between the development of such cancer and the appearance of NGAL in the urine.

46. Blaser relates to detecting the level of NGAL (8 $\mu\text{g/L}$ = 8 ng/mL) in pooled urine samples in healthy donors over a 24 hour period. (See the last sentence in section 2.4 Preparation of the samples, on page 139). Thus, this reference implies only that some level of NGAL is found in the urine of alleged “healthy” persons. Blaser does not report or suggest that the kidney is a source of the observed NGAL, or has even accessed the urine through the kidneys. Indeed, the source of NGAL in the urine of Blaser may be completely unrelated to the kidney (such as, the bladder). It is well known that the urine contains very few proteins (dozens perhaps), compared to the thousands of known proteins expressed within the tissues of the kidney itself, and that source of a protein appearing in the urine cannot easily be predicted as having been sourced from the kidney, as opposed to the bladder, prostate (in men), vaginal tissue (in women),

blood, or other source. Since the site of origin of urinary NGAL from Blaser is simply not known, Blaser's association with Matthaeus is unsupportable, and - at best - improper hind-sight.

47. The Office Action (page 10 lines 11-17) goes on to state that "Ohlsson et al found that *greatly elevated NGAL levels are strongly correlated with decreased renal function* (emphasis in the rejection)", and that "(t)aken together with the finding of Matthaeus 1 or 2, it would have been obvious to detect NGAL for the purpose of diagnosing renal dysfunction since the reference established that NGAL is specifically elevated in this disease condition, and further in view of the general knowledge of one skilled in the art at the time of the invention that markers changed in response to disease can be used as biomarkers for diagnosis of the disease."

48. We do not agree with this characterization of Ohlsson et al. Ohlsson et al states specifically that "greatly elevated NGAL levels were seen in our patients. However, there was a strong correlation with decreased renal function" (page 531, the left column, last paragraph). The exact phrasing of the statement made in Ohlsson et al is important. Ohlsson et al made a general statement that their patients had elevated levels of NGAL. When the authors noted that the NGAL level correlated with elevated levels of cystatin C (the renal function markers used by the authors in the study), they "corrected" the NGAL result dividing the NGAL level by the cystatin C level, to normalize the result with "renal function". It is clear that Ohlsson et al taught that cystatin C was the renal function marker, and that in these particular patients, the level of NGAL showed a correlation with renal function. This was not to convey that the level of NGAL correlates with renal function generally.

49. We refer the Examiner to the 132 Declaration submitted by Prasad Devarajan in the co-pending application 11/096,113, which was submitted with the response and the RCE filed on February 20, 2007, related to the Ohlsson et al reference. We append a copy of such 132 Declaration (hereinafter, "Ohlsson Declaration") herewith as Appendix A.

50. The Ohlsson Declaration mentioned above stated Dr. Devarajan's opinion that the authors of Ohlsson et al (2003) clearly attribute the result (an elevated NGAL levels that correlated with decreased renal function) to neutrophil activity caused by some factor other than

renal dysfunction, for which cystatin C was their marker, since the authors normalize the NGAL level to cystatin C, and they attribute the increase in neutrophil degranulation to the treatment of the DC group with immunosuppressive medication. (It is well known that neutrophils are a major source of MGAL in the blood, the sampling source in Ohlsson et al).

51. We also note that Ohlsson et al describes “renal dysfunction” as opposed to “renal injury”. Since it is well known to person of ordinary skill in the art that a downturn in renal function trails substantially an earlier, undetected kidney injury (typically by a couple of days), even the correlation of the level of NGAL with kidney dysfunction is not predictive of a biomarker of the early onset of a renal tubular cell injury.

52. We also believe that the detection of the level of NGAL in a serum sample would have revealed nothing to a person of ordinary skill concerning the level of NGAL in urine, not unlike the skilled person’s understanding that increases in the level of creatinine in the serum are not predictive of an increase in the level of creatinine in urine.

53. We also noted that the Office Action refers to David et al (US 4,376,110), which relates to now well-known ELISA techniques. David makes no mention of renal injury or disease, or its detection via the urine, or even the “renal”, “kidney”, “disease”, “injury” or “urine”. David et al does teach that the technique reduced significantly the time for running the assay.

54. The Office Action also cites Muramatsu et al. Muramatsu et al teach detecting the presence of Cyr61 protein extracted out of mouse urine onto heparin beads, to detect an ischemic renal injury. Muramatsu et al makes no reference to NGAL. While a person of ordinary skill in the art might identify and appreciate parallels between Cyr61 in *Muramatsu and our NGAL invention*, the rejection greatly exaggerates any association that a person of ordinary skill in the art might possibly make between urinary Cyr61 in Muramatsu and the detection of NGAL and MMP-9 in the injured proximal tubuli of Matthaeus after 24 and 48 hours. Other than a rat/mouse model of ischemic renal injury (although Matthaeus do not describe with any specificity the mode of inducing ischemia), there are substantial differences between the

Muramatsu and Matthaeus reference. The authors were looking at different proteins (Cyr-61 versus NGAL and MMP-9), in different locations (in urine, versus in the kidney), at different times (within hours, versus after 24 hours), and for different reasons (detecting ischemic injury, versus detecting the kidney's response to injury. Other than the "who" (the mouse), the "what", "when", "where" and "why" between Muramatsu and Matthaeus are different.

55. The Office Action states that Sanicola-Nadel (US 6,664,385) teaches use of KIM-1 protein in a diagnostic method, in the abstract and at column 1 lines 49-55. We looked at the patent, and we note that the only mention of KIM-1 protein and diagnostic methods is at col 2 line 66 to column 3 line 21, and at column 17 lines 34-41.

56. The Office Action goes on (page 16 lines 10 – 15) to allege that Sanicola-Nadel et al teach that KIM-1 protein is selectively upregulated at any time within one week following any insult that results in injury to kidney tissue, mentioned at column 5 lines 17-27.

57. We do not agree with this characterization of Sanicola-Nadel. Sanicola-Nadel at col 5 lines 27 refers to the time of the selective upregulation of *KIM-1* mRNA, and not to the expression or appearance of KIM-1 protein *per se*. Moreover, a person of ordinary skill in the art would understand that Sanicola-Nadel speculates, rather than teaches, that such upregulation of mRNA "might be identified" at 10 hours. While such time is "within one week", the time course for appearance of KIM-1 protein within the urine or serum is not addressed at all.

58. We also note that the detection of KIM-1 protein is from a homogenized kidney that is excised 24 hours and 48 hours after unilateral kidney ischemia (see column 17 line 66 to column 18 line 8). Specifically, kidney homogenates of *contralateral and post-ischemic kidneys* were examined following a 40 minute clamping of *the renal artery and vein of a single kidney* for each rat. It is well known to persons skilled in the art that unilateral renal ischemia does not necessarily result in the development of acute renal failure, which we also expressly teach in the instant application (our paragraph [0085]). Traditional markers of ischemic renal injury, including serum creatinine, are not usually elevated in a unilateral ischemic rat or mouse model. Therefore, a person of ordinary skill in the art would understand that the authors speculate that

KIM-1 might be found in either the urine or the serum of the disclosed rat models of Sanicola-Nadel, since no urinary or serum renal marker was known at the time of Sanicola-Nadel or at the time of the present invention, had been shown to appear within 24 hours of an ischemic renal injury, and no actual showing of KIM-1 in urine or serum is actually shown.

59. We would not predict that the results observed for KIM-1 protein would be applicable to NGAL for numerous reasons. KIM-1 protein is quite different from NGAL in properties and size; for example, NGAL is a secreted whole protein, while in the case of KIM-1, only a small ectodomain of the protein is proteolytically processed and appears in the urine. That is to say, any appearance of the ectodomain of the KIM-1 protein in urine involves a kinetically-driven, time-consuming biological process, with an unpredictable duration before its appearance in the urine. NGAL at the time of the present invention was generally associated with the degranulation of activated neutrophils. Any prediction or suggestion based upon KIM-1 protein could not carry over to NGAL, and *visa versa*.

We further declare that all statements made of our knowledge are true and that all statements made on information and belief are believed to be true; further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001 and may jeopardize the validity of the application or any patent issuing thereon.

228,09

Date _____

Am Bm

Jonathan M. Barasch

Date _____

Prasad Devarajan

18 USC 1001: "Whoever in any matter within the jurisdiction of any department or agency of the United States knowingly and willfully falsifies, conceals or covers up by any trick, scheme, or device a material fact, or makes any false, fictitious or fraudulent statements or representations, or makes or uses any false writing or document knowing the same to contain any false, fictitious or fraudulent statement or entry, shall be fined not more than \$10,000 or imprisoned not more than five years, or both."

KIM-1 might be found in either the urine or the serum of the disclosed rat models of Sanicola-Nadel, since no urinary or serum renal marker was known at the time of Sanicola-Nadel or at the time of the present invention, had been shown to appear within 24 hours of an ischemic renal injury, and no actual showing of KIM-1 in urine or serum is actually shown.

59. We would not predict that the results observed for KIM-1 protein would be applicable to NGAL for numerous reasons. KIM-1 protein is quite different from NGAL in properties and size; for example, NGAL is a secreted whole protein, while in the case of KIM-1, only a small ectodomain of the protein is proteolytically processed and appears in the urine. That is to say, any appearance of the ectodomain of the KIM-1 protein in urine involves a kinetically-driven, time-consuming biological process, with an unpredictable duration before its appearance in the urine. NGAL at the time of the present invention was generally associated with the degranulation of activated neutrophils. Any prediction or suggestion based upon KIM-1 protein could not carry over to NGAL, and *visa versa*.

We further declare that all statements made of our knowledge are true and that all statements made on information and belief are believed to be true; further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001 and may jeopardize the validity of the application or any patent issuing thereon.

Date

12/9/09

Date

Jonathan M. Barasch

Prasad Devarajan

Prasad Devarajan

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :
PRASAD DEVARAJAN et al : Confirmation No. 5802
Serial No.: 11/096,113 : Group Art Unit 1641
Filed: March 31, 2005 : Examiner FOSTER, Christine E.
For: METHOD FOR EARLY DETECTION OF
RENAL INJURY

DECLARATION UNDER 37 CFR 1.132
REGARDING OHLSSON ET AL. (2003)

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Prasad Devarajan, do hereby declare as follows:

1. I am a co-inventor of the subject matter described and claimed in the above-identified patent application.

2. I hold an undergraduate degree in Biology and a Medical Doctor (M.D.) degree from Bombay University, India. I also completed graduate research in renal disease in the Department of Physiology, and a residency in Pediatrics at SUNY at Stony Brook, NY. I also completed a fellowship in Nephrology at Yale University, and completed an NIH-sponsored research fellowship in renal disease at Yale.

3. I have been conducting research in the field of renal disease since 1985. I am presently the Louise M. Williams Endowed Chair, Professor of Pediatrics, Professor of Developmental Biology, Director of Nephrology and Hypertension, Director of Nephrology Clinical Laboratories, CEO of Dialysis Unit, at Cincinnati Children's Hospital Medical Center and the University of Cincinnati School of Medicine, Cincinnati, OH.

I am an expert reviewer of grant applications in the field of renal diseases for the NIH and several other national and international organizations. I am an expert reviewer of

publications submitted to more than 20 scientific and medical journals in the field of renal disease. I am a member of the Editorial Board of key journals in the field of renal disease. I am on the Advisory Board and Research Committees of the American Society of Nephrology, American Society of Pediatric Nephrology, International Acute Kidney Injury Network, and the National Institutes of Health in the field of renal disease.

4. I have read and am familiar with the Final Office Action in the present application issued on October 19, 2006, and have read and as familiar with the publication reference of Ohlsson et al. (2003).

5. I understand Ohlsson et al. as describing the study of patients with ANCA-associated systemic vasculitis (SV Patients) in remission, to understand if proteinase 3 (PR3) levels are increased, and if this increase is associated with neutrophil degranulation (by measuring NGAL), with renal function (by measuring cystatin C), and with inflammation (by measuring CRP, IL6, and sTNF α 1). The authors studied a group of SV Patients and recorded six years later the status of the SV Patient group (p. 529 "Patient material"). Of the 59 SV Patients originally sampled as having ANCA-associated systemic vasculitis, an unknown number (perhaps up to 15 from the "smouldering" group) were identified as having "renal failure", based on cystatin C level information, as shown in Table 1. The cystatin C was the only assay used to measure kidney function. Interestingly this sub-group is also known as "severe outcomes" and includes persons who died due to vasculitis complications during the 6 years follow-up (see the footnote under Table 1).

6. The paragraph in Ohlsson et al is titled "PR3 versus neutrophil activation and degranulation", and reads in its entirety:

"Degranulating activated neutrophils are also a possible PR3 source and NGAL is a marker of neutrophil activation and degranulation. Greatly elevated NGAL levels were seen in our patients. There was, however, a strong positive correlation with decreased renal function ($r=0.8$. $P<0.001$) (Fig. 3). After correction for this (the PR3 values were divided by the cystatin C values, giving us the parameter NGAL/C) slightly increased levels (110, 42-340, $n=59$) were still seen compared with healthy blood donors (81, 38-130, $n=25$), but not compared with the diseased controls (120, 57-260, $n=48$) (Fig. 4)." (underlined emphasis added)

It appears to me that the statement “the *PR3 values* were divided by the cystatin C values, giving us the parameter NGAL/C” is a typographic error. I believe that the authors intended the highlighted phrase to read “*NGAL values*”, since Table 4 defines NGAL/C as NGAL divided by cystatin C.]

7. Furthermore, the Abstract, which states “The raised NGAL levels correlated strongly with decreased renal function ($r=0.8$, $P<0.001$).” The Abstract goes on to state “After correcting for this, slightly increased levels were observed compared with healthy blood donors...but not compared with the disease controls.”

8. In my opinion, the authors would “correct” for a raised level of NGAL, by dividing by cystatin C, which is the only marker for renal function, in order to factor out and exclude the renal function from the appearance of NGAL. In my opinion, the authors did not consider that the level of NGAL was a direct result of renal function, but that the level of NGAL was being impacted by the renal function, as measured by cystatin C. It appears to me that at all times, these authors considered NGAL to be nothing more than a marker for neutrophil degranulation. The authors were determined to “factor out” the renal function factor, by normalizing the NGAL levels to the level of cystatin C.

9. In my opinion, the authors did not recognize, and were not recognizing, NGAL as a marker for renal function.

10. On page 534, left column, first paragraph, the authors discuss the results of the work. First the authors note that “(e)levated NGAL and sTNFrl levels correlated strongly with decreased renal function”.

11. The authors stated that sTNFrl is a marker for inflammation. I am not aware of any reference that teaches that sTNFrl a marker for renal function, and I believe that it is not a marker for renal function.

12. Having no prior basis to expect that sTNFrl is a marker for renal function, I would not have concluded, and I do not believe that a person of ordinary skill in the art would

understand, that sTNF α is a marker for evaluating renal tubule cell injury, based on this reported strong correlation with decreased renal function.

13. At the time of Ohlsson et al., I am unaware of any reference that taught that NGAL was a marker for renal function. By the same reasoning above regarding sTNF α , I do not believe that a person of ordinary skill in the art, in view of Ohlsson et al., would understand that renal tubular cell injury can be evaluated based on the level of NGAL, and that NGAL is a marker of renal function.

14. The discussion in Ohlsson et al. continues:

“After correction [by dividing by the renal function marker, cystatin C], only moderate increased values were seen for NGAL/C compared to HBD [healthy blood donor] and not at all compared with DC [diseased control]. In the DC group, but not in the vasculitis patients, NGAL/C and PR3 showed a significant positive correlation [data in Fig. 4]. This would indicate that the slightly elevated PR3 levels in the DC group could be explained by, for example, a therapy-related neutrophil influence, whereas there must be another explanation for this in the vasculitis patients.” (emphasis added)

15. In my opinion, the authors are saying that, after normalized the level of NGAL to cystatin C, their only renal function marker, the higher level of NGAL in the Disease Control (DC) group is explained by an increase in neutrophil degranulation, perhaps caused by the treatments of the DC group with immunosuppressive medication (page 529, right column, end of the initial paragraph). My opinion is further supported by the Ohlsson et al. authors’ concluding statement at page 534, left column, last paragraph, that significantly raised levels of PR3 in plasma from patients with small vessel vasculitis were “not due to decreased renal function, ongoing inflammation or neutrophil activation. (emphasis added)” The authors’ conclusion appears to me to be based on using cystatin C as a marker for decreased renal function, and NGAL as a marker of neutrophil activation.

16. Ohlsson et al. appears to establish a correlation between level of NGAL with renal function. Figure 3 may show a correlation between log NGAL and log cystatin C. Nevertheless, it is my professional belief that the authors clearly attribute this result to

neutrophil activity, for which NGAL is their marker. I do not believe that the authors of Ohlsson et al. attribute the presence and level of NGAL as a direct result of renal injury.

17. While Ohlsson et al. state that NGAL correlated strongly with decreased renal function, it is my opinion that the reference clearly attributes the level of NGAL to some factor other than renal injury. First, the authors normalize the NGAL level to cystatin C, which is their only renal function marker. Second, the authors attribute the increase in neutrophil degranulation to the treatment of the DC group with immunosuppressive medication.

18. In my opinion, the only reasonable interpretation of Ohlsson et al. is that renal function is assessed using cystatin C. In 2002, Dharnidharka et al published a comprehensive meta-analysis of 46 articles that compared the efficacy of cystatin C with a standard measurement of kidney function. This analysis clearly documented that cystatin C is the most superior marker of kidney function, in subjects with normal kidney function as well as in various disease states (Dharnidharka VR, Kwon C and Stevens G: *Serum cystatin C is superior to serum creatinine as a marker of kidney function*, American Journal of Kidney Diseases 40:221-226, 2002; copy attached).

19. Further, Ohlsson et al. disclose that the levels of NGAL, PR3, cystatin C and other molecules were determined in samples from patients having ANCA-associated systemic vasculitis and control subjects, that the systemic vasculitis condition associated with such patients had been in a stable phase for at least six years, and that the disease control subjects (DCs) all had kidney transplants and were on immunosuppressive medication for an unspecified, but presumably long time.

20. The time between any event associated with the systemic vasculitis and obtaining the samples of blood from the patients was at least 6 years. While the time between the kidney transplantation of the disease control group and the blood sampling is not stated, in my opinion, any such time would have to be substantially longer than 24 hours. Thus, I do not believe that Ohlsson et al. disclose nor suggest, nor make obvious, sampling of the blood from a mammal within 24 hours of an event that causes or places the mammal at risk of


developing a renal tubular cell injury, as provided in some of the claims of our patent application.

21. Therefore, it is my opinion that the Ohlsson et al. reference neither discloses nor suggests to a person of ordinary skill in the art that the level of NGAL can be used to evaluate renal tubular cell injury status.

22. Ohlsson and others published a second paper in 2005 (S. Ohlsson, T. Hellmark, K. Pieters, G. Sturfelt, J. Wieslander and M. Segelmark, *Increased monocyte transcription of the proteinase 3 gene in small vessel vasculitis*, Clinical and Experimental Immunology, 141: 174-182; copy attached), which made reference (page 175, left column) to the previous Ohlsson et al. reference (2003) cited by the Examiner. In the 2005 paper, the authors stated that the 2003 paper ruled out decreased renal function as the cause of raised circulating levels of PR3 using cystatin C as a marker of glomerular filtration (GFR), and that NGAL was measured as a marker of neutrophil degranulation. It appears that these same authors, also persons of skill in this art, had not considered that their earlier work had taught or suggested that the level of NGAL can be used to evaluate renal tubular cell injury status.

I further declare that all statements made of my knowledge are true and that all statements made on information and belief are believed to be true; further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001 and may jeopardize the validity of the application or any patent issuing thereon.

FEBRUARY 13, 2007
Date


Prasad Devarajan

18 USC 1001: "Whoever in any matter within the jurisdiction of any department or agency of the United States knowingly and willfully falsifies, conceals or covers up by any trick, scheme, or device a material fact, or makes any false, fictitious or fraudulent statements or representations, or makes or uses any false writing or document knowing the same to contain any false, fictitious or fraudulent statement or entry, shall be fined not more than \$10,000 or imprisoned not more than five years, or both."



American Journal of
Kidney Diseases

META-ANALYSIS

Serum Cystatin C Is Superior to Serum Creatinine as a Marker of Kidney Function: A Meta-Analysis

Vikas R. Dhamidharka, MD, Charles Kwon, MD, and Gary Stevens, PhD

• **Background:** Serum cystatin C (Cys C) has been proposed as a simple, accurate, and rapid endogenous marker of glomerular filtration rate (GFR) in research and clinical practice. However, there are conflicting reports regarding the superiority of Cys C over serum creatinine (Cr), with a few studies suggesting no significant difference. **Methods:** We performed a meta-analysis of available data from various studies to compare the accuracy of Cys C and Cr in relation to a reference standard of GFR. A bibliographic search showed 46 articles until December 31, 2001. We also retrieved data from eight other studies presented and published in abstract form. **Results:** The overall correlation coefficient for the reciprocal of serum Cys C ($r = 0.816$; 95% confidence interval [CI], 0.804 to 0.826) was superior to that of the reciprocal of serum Cr ($r = 0.742$; 95% CI, 0.726 to 0.758; $P < 0.001$). Similarly, receiver operating characteristic (ROC)-plot area under the curve (AUC) values for 1/Cys C had greater identity with the reference test for GFR (mean ROC-plot AUC for Cys C, 0.926; 95% CI, 0.892 to 0.960) than ROC-plot AUC values for 1/Cr (mean ROC-plot AUC for serum Cr, 0.837; 95% CI, 0.796 to 0.878; $P < 0.001$). Immunonephelometric methods of Cys C assay produced significantly greater correlations than other assay methods ($r = 0.846$ versus $r = 0.784$; $P < 0.001$). **Conclusion:** In this meta-analysis using currently available data, serum Cys C is clearly superior to serum Cr as a marker of GFR measured by correlation or mean ROC-plot AUC.

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INDEX WORDS: Cystatin; creatinine (Cr); glomerular filtration rate (GFR); kidney function; meta-analysis.

THE BEST GLOBAL index of renal function is glomerular filtration rate (GFR).¹ Inulin clearance has been considered the reference standard for GFR measurement because inulin fulfills all criteria for an ideal GFR marker (ie, stable production rate and circulating levels not affected by other pathological changes, freely filtered at the glomerulus without tubular reabsorption or secretion). More recently, clearances of radioisotope-labeled or nonlabeled trace quantities of chromium 51-EDTA (⁵¹Cr-EDTA), technetium 99-diethylenetriamine pentacetic acid (⁹⁹Tm-DTPA), iothalamate, or iohexol have shown greater than 97% identity and have been accepted as accurate substitutes for inulin clearance.^{2,3} However, all these techniques are labor and time intensive and thus not ideal for clinical practice or large-volume clinical research. Endogenous filtration markers are less complex and

provide more rapid results. The most commonly used endogenous filtration markers in clinical practice are serum creatinine (Cr) value or its reciprocal, alone or in conjunction with 24-hour urine collections for creatinine clearance. However, many factors limit the accuracy of these markers as measures of GFR, including reliabil-

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0272-6386/02/4002-0001\$35.00/0
doi:10.1053/ajkd.2002.34487

ity of urine collection, influence of body mass, dietary intake, aging, and analytic problems with assay methods. Thus, there has been an ongoing search for suitable alternative endogenous markers of GFR.

Cystatin C (Cys C) is a cationic nonglycosylated low-molecular-weight (13,359 kd) cysteine proteinase.^{4,5} This compound is stably produced by all nucleated cells in constitutive fashion and meets criteria for a GFR marker described previously. Use of serum Cys C value (or its reciprocal) as a measure of GFR was proposed in 1985.^{6,7} Since that time, multiple studies have been performed to evaluate the accuracy of Cys C level as a marker of GFR. Most of these studies have shown a high degree of correlation for the reciprocal of Cys C (1/Cys C) with the comparative GFR reference standard.⁸⁻¹⁰ In addition, in some studies, receiver operating characteristic (ROC) curves for Cys C were significantly superior to curves for serum Cr.¹¹⁻¹⁵ Conversely, a few studies could not show a significant difference between Cys C and Cr, although there was a trend for greater accuracy with Cys C by either greater ROC-plot area under the curve (AUC) values or greater correlation coefficients.¹⁶⁻²⁰ These conflicting results have precluded the widespread use of Cys C level as a measure of GFR in clinical research and clinical practice. Coll et al²¹ postulated the existence of a type II error in studies that did not show a significant difference, ie, acceptance of the null hypothesis because of insufficient sample size. This would need assessment by a very large single study (>1,000 patients) or a meta-analysis of several existing studies.²¹

In this study, we performed such a meta-analysis and show that Cys C level is a significantly better indicator of GFR based on analysis of both ROC-plot AUC data and correlation coefficients.

METHODS

A bibliographic search was performed for all published articles pertaining to the use of serum or plasma Cys C level as a marker of renal function or GFR. As of December 31, 2001, we identified 46 articles that compared the efficacy of Cys C with a standard method of GFR measurement, such as clearance of inulin or the tracers ⁵¹Cr-EDTA, ⁹⁹Tm-DTPA, iothalamate, or iohexol. Review articles were included only if original and previously unpublished data were presented.

In addition, we also included in this analysis data from seven conference abstracts from annual meetings of the American Society of Nephrology published in abstract form only until December 2001^{14,20,22-26} and one set of data presented at a symposium in October 2001 (T. Larsen, unpublished data). From among all studies analyzed, 33 studies compared the correlation coefficient *r* with that of serum Cr.^{6-8,10-20,22-48}

However, correlation coefficients may reflect only a linear association and not always translate into agreement or diagnostic accuracy. It is possible for a test to show high correlation with a reference standard and yet consistently overestimate or underestimate the true value of the measure. Thus, more meaningful tests are comparisons of sensitivity, specificity, and positive and negative predictive values. These are highly dependent on normal cutoff values chosen for the test under scrutiny. Our meta-analysis showed a widely disparate range of cutoff values for Cys C (0.90 to 1.40 mg/L), thus precluding meaningful analysis of these parameters among studies.

Another method for assessment of diagnostic accuracy of a new test is ROC analysis, in which the sensitivity of the test is plotted against the 1-specificity over all possible decision values and ROC-plot AUC measured against the reference standard (ROC-plot AUC of reference standard = 1.00). ROC-plot AUC analysis is an effective way to determine which test offers the best overall clinical performance and is used widely to show limits of a test's ability to discriminate state of health versus abnormality.⁴⁹⁻⁵¹

We identified 16 articles that compared ROC curves for Cys C with the reference standard,^{11-15,18,19,28,31,32,41,43,48} and 14 of these published data on ROC-plot AUC values for Cys C and Cr with the reference standard.^{11-14,18,19,28,31,32,43,48} Subject populations included adults and children and healthy volunteers and patients with varying degrees of renal impairment caused by a diverse group of conditions.

Statistical analysis was performed using SAS version 8.1 software (SAS Institute, Cary, NC). The null hypothesis was stated as follows: there is no difference between correlation coefficients and ROC-plot AUCs for serum Cys C and Cr compared with the reference standard for GFR. Correlation coefficients (*r*) were analyzed using Fisher's *z* transformation, in which $z = 0.5 \log (1 + r/1 - r)$, with transformed data assumed to have a normal distribution. The overall correlation and corresponding confidence intervals (CIs) for both Cys C and Cr were obtained by calculating a weighted average of *z*-transformed values and subsequent back transformation. Correlation and ROC-plot AUC data were compared using Student's *t*-test and 95% CI calculations. *P* less than 0.05 was considered significant.

RESULTS

Cys C Has a Greater Correlation Coefficient Than Cr

There were 54 data sets incorporating 4,492 subject samples included in this meta-analysis. Of these, we first compared 36 data sets for Cys C correlation coefficients with 29 data sets for serum Cr. The range of correlation coefficients for both 1/Cys C and 1/Cr is shown in Fig 1A. As

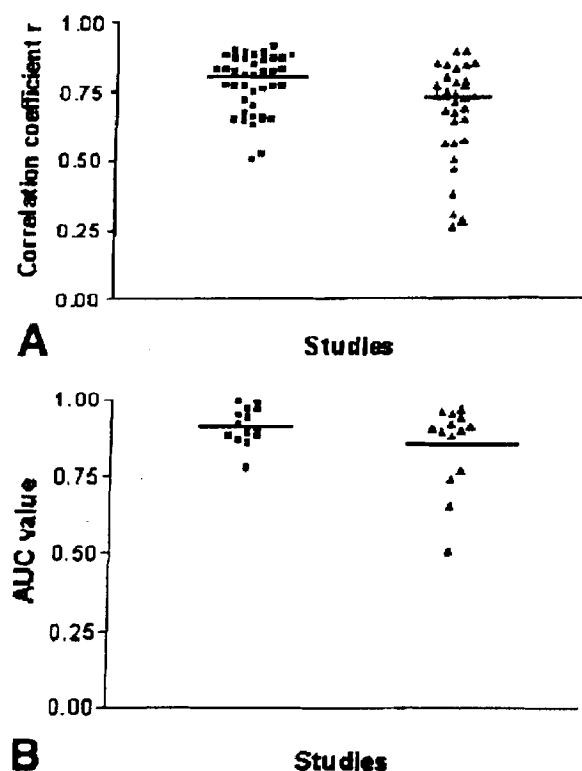


Fig 1. (A) Scatter plot of correlation coefficients (r) for (■) 1/Cys C and (▲) 1/Cr from studies analyzed. Horizontal line represents the cumulative mean r of all studies. (B) Scatter plot of ROC-plot AUC values for Cys C and Cr from 14 data sets. Horizontal line represents the cumulative mean. Note that meta-analysis was performed on 11 data sets that published mean ROC-plot AUC + SE values. Three studies shown in this graph did not publish SEs.

listed in Table 1, the overall coefficient of correlation r was significantly greater for 1/Cys C (mean $r = 0.816$) in comparison to 1/serum Cr (mean $r = 0.742$; $P < 0.001$). There was no overlap between 95% CIs (Cys C, 95% CI, 0.804 to 0.826; Cr, 95% CI, 0.726 to 0.758).

Cys C Has a Greater ROC-Plot AUC Identity Than Cr

We further identified 14 studies with 1,643 subject samples that published ROC curves and ROC-plot AUC data with sample sizes. Three of these data sets did not publish SE values and were excluded from statistical meta-analysis. The final analysis was performed on 11 data sets with 997 subjects. ROC-plot AUC data for 1/Cr in different studies showed a much greater divergence of values, ranging from 0.5070 to 0.9600,

whereas ROC-plot AUC values for 1/Cys C showed a much smaller dispersion (Fig 1B).

As listed in Table 1, the combined ROC-plot AUC for Cys C by meta-analysis was significantly greater (mean, 0.926; 95% CI, 0.892 to 0.960) in comparison to the ROC plot AUC for serum Cr (mean, 0.837; 95% CI, 0.796 to 0.878; $P < 0.001$). Similar to data for correlation coefficients, there was no overlap in 95% CIs for ROC-plot AUC values. ROC-plot AUC comparisons and meta-analyses provide the strongest data in support of the superiority of Cys C over Cr as a measure of GFR.

Immunonephelometric Assay for Cys C Is Superior to Other Methods

The different studies that we included in this analysis spanned a period of 15 years and used several different assay methods for Cys C, such as enzyme-amplified single radial immunodiffusion, sandwich enzyme immunoassay, and immunoturbidometric assays.^{6,7,15,52} More recent reports used either an immunoturbidometric or immunonephelometric method.^{33,36,40,42} A recent report suggests that the immunonephelometric assay is superior to the immunoturbidometric assay for measurement of Cys C.¹³ To assess whether differences in methods account for conflicting reports of Cys C superiority, we performed a meta-analysis of correlation coefficients of studies that used immunonephelometric assays versus other techniques. As listed in Table 2, there were 14 studies (1,698 subjects) with published r values that used immunonephelometric assays and 21 studies with published r values that used other assay methods (1,953 subjects). The mean r for immunonephelometric assay studies was significantly greater (mean $r = 0.846$; 95% CI, 0.832 to 0.859) than the mean r for other

Table 1. Comparison of Correlation Coefficients and ROC-Plot AUC Values for Reciprocals of Cys C and Cr to GFR Reference Standard

Parameter	Test	No.	k*	Mean (95% CI)
Correlation coefficient r	Cys C	3,703	36	0.816 (0.804-0.826)†
	Cr	3,101	29	0.742 (0.726-0.758)
ROC-plot AUC	Cys C	997	11	0.926 (0.892-0.960)†
	Cr	997	11	0.837 (0.796-0.878)

*Number of data sets.

† $P < 0.001$ in comparison to Cr.

Table 2. Comparison of Correlation Coefficients Based on Cys C Assay Method

Assay	No.	k*	Lower Limit	Mean	Upper Limit
Immunonephelometric	1,698	14	0.832	0.846	0.859
Others	1,953	21	0.766	0.784	0.801

*Number of data sets.

assay-based studies (mean $r = 0.784$; 95% CI, 0.766, 0.801; $P < 0.001$). Statistical significance was evident by the lack of overlap in 95% CIs. We could not compare ROC-plot AUC data because there were too few studies with available data on ROC-plot AUC curves to determine whether the method of Cys C assay used influenced the ROC-plot AUC.

DISCUSSION

The need for a simple, accurate, and minimally invasive marker of GFR has been a limiting factor in clinical nephrology research and practice. Although serum Cr is widely used as such a marker, its limitations are well known. More accurate methods, such as radiolabeled tracer clearances, are invasive, may involve radiation, and require several hours to perform. Many low-molecular-weight serum proteins were proposed as suitable endogenous markers of GFR, including β_2 -microglobulin and retinol-binding protein.^{6,16,44} Among these low-molecular-weight proteins, Cys C has received the most interest. This interest has increased in recent years with the advent of newer technological methods that render the assay easier to run.

However, several studies have questioned the value of Cys C. In these studies, the correlation coefficient for 1/Cys C was either not significantly better^{6,7,40} or even lower than the coefficient for 1/Cr.^{19,20} Potential criticisms of these studies have included: (1) the choice of reference standard for GFR (such as the less accurate creatinine clearance), (2) use of inappropriate units of GFR (milliliters per minute [milliliters per second] instead of milliliters per minute per 1.73 m²), and (3) insufficient sample size, leading to a type II error. Among studies that compared ROC-plot AUC data, only two showed greater ROC-plot AUC values for 1/Cr than 1/Cys C,^{19,43} whereas the rest showed 1/Cys C to

be superior. In both these studies, ROC-plot AUCs for both 1/Cr and 1/Cys C exceeded 0.900.^{19,43} These high ROC-plot AUC values for 1/Cr were not reproducible in other studies. Thus, 1/Cys C was not found to be inaccurate in either of these studies, although superiority to Cr could not be shown. In addition, both studies used an immunoturbidometric method, rather than an immunonephelometric method.

In the present study using a meta-analysis of combined data from all studies, we clearly show that Cys C shows superior correlation coefficients and greater ROC-plot AUC values than serum Cr. These conclusions were reached without the need to weight the studies by quality of design, such as for choice of reference GFR standard or accuracy of units. Limiting our analysis to studies that used an acceptable method of GFR assay and milliliters per minute per 1.73 m² did not change our results or conclusions.

We do not directly address the issue of the accuracy of serum Cys C level by itself as a substitute for established reference tests for GFR measurement. However, it is worth noting that in all studies in which ROC-plot AUC data were published, the reference standard test for GFR used a clearance technique, such as inulin or radiolabeled tracer. On these studies, the mean ROC-plot AUC for 1/Cys C was 0.924, with a small range of dispersion. Although we did not analyze for degree of renal failure separately, previous studies have shown that correlation of GFR with 1/Cys C increases with greater degrees of renal failure. Thus, we believe that the reciprocal of Cys C accurately reflects true GFR across a spectrum of renal function and therefore would be an adequate substitute for radiotracer clearance studies, as well.

With the accuracy of Cys C established, determination of its utility as a measure of GFR in clinical practice rests on its cost-effectiveness over Cr level. However, for clinical research, advantages of Cys C over clearance techniques already are apparent. This test is considerably less labor and time intensive than clearance techniques and will cause less patient inconvenience and morbidity. Compared with radiolabeled tracers, Cys C is less expensive and does not pose radiation hazards to subjects or personnel. Thus, with greater than 92% identity of Cys C ROC-plot AUC to that of reference tests for GFR over

all possible values, there is justification to use Cys C as the future marker of GFR in clinical research.

In conclusion, our meta-analysis confirms the superiority of 1/Cys C over 1/Cr with respect to GFR measures, by both superior correlation coefficients and greater ROC-plot AUC values. Lack of superiority over 1/Cr in previous studies may reflect a type II error or differences caused by assay methods.

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Increased monocyte transcription of the proteinase 3 gene in small vessel vasculitis

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Summary

Proteinase 3 (PR3) is a pleiotropic and destructive serine protease and it is also a major target for autoantibodies in systemic small vessel vasculitis. We have shown recently that patients in stable remission have increased circulating levels of PR3, independent of autoantibody titre, inflammation, neutrophil degranulation and renal function. Here we explore the possibility of increased PR3 gene transcription. RNA was purified from peripheral blood monocytes from vasculitis patients and controls. Specific mRNA was measured by *TaqMan* real-time polymerase chain reaction (PCR). The monocyte-like cell lines THP-1 and U937 and human peripheral blood monocytes from healthy controls were stimulated with cytokines and lipopolysaccharide (LPS) for different time periods. PR3 protein was measured in plasma with enzyme-linked immunosorbent assay (ELISA). The median result for PR3 mRNA was 9.6 (1.8–680) for 22 patients, compared to 1 (0.1–2.8) for the 15 healthy controls. Elastase expression was also significantly increased, whereas myeloperoxidase and interleukin-8 were not. Stimulation of monocytes with tumour necrosis factor (TNF)- α , interferon (IFN)- γ or LPS did not result in any increase of PR3 or elastase transcription, whereas interleukin (IL)-8 transcription was increased 10-fold. Circulating monocytes from patients with systemic vasculitis display increased PR3 gene transcription compared to healthy controls and patients with systemic lupus erythematosus (SLE). This may be important for the development of vasculitis. Our results do not favour a role for cytokines, antineutrophil cytoplasmic antibodies (ANCA) or immunosuppressive medication in the upregulation of PR3 transcription in vasculitis.

Keywords: ANCA, monocytes, proteinase 3, real time PCR, systemic vasculitis

Accepted for publication 21 March 2005

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Introduction

Wegener's granulomatosis and microscopic polyangiitis are systemic small vessel vasculitides of unknown aetiology. Due to the strong association with autoantibodies reacting with granule constituents of neutrophils and monocytes, they are often referred to as ANCA-associated small vessel vasculitis (AASV; ANCA: antineutrophil cytoplasmic antibodies). The major ANCA target antigens are myeloperoxidase (MPO) and proteinase 3 (PR3) [1].

PR3 is a 29-kDa serine protease stored in granules of neutrophils and monocytes. It is also present on the surface of primed or apoptotic human neutrophils in a bioactive form [2]. Extracellularly, PR3 is inhibited through binding to

serine protease inhibitors (SERPINS), the most abundant in plasma being α 1-antitrypsin (α 1-AT). The substrates of PR3 include several extracellular matrix components, and the protease has been shown to produce emphysema in hamsters when installed intratracheally [3]. Besides its proteolytic effects, PR3 has influence on the proliferation of granulopoietic progenitor cells [4] and it can induce apoptosis in endothelial cells [5]. PR3 is also involved in cytokine activation, chemokine activity amplification and processing of cytokine binding proteins, indicating a regulatory role in inflammatory processes [6].

Few genetic polymorphisms have been associated reproducibly with AASV. An exception is the PiZ deficiency allele of α 1-AT that is associated with an increased risk to develop

AASV. This association has been reported in studies from France, Austria, Holland, United States, England and Sweden [7]. In our cohort we also found the PiZ allele to be linked to disease extension and a worse prognosis. These findings suggest increased protease activity to be part of the pathogenesis.

We have recently published findings of raised circulating levels of PR3 in AASV patients in remission or with chronic smouldering disease activity [8]. These high levels were not an effect of general inflammation, as there was no correlation between PR3 and C-reactive protein (CRP) or interleukin (IL)-6; nor was there any correlation with clinical disease activity, using Birmingham Vasculitis Activity Score (BVAS). We could also rule out decreased renal function, using relevant disease controls and cystatin C as a marker of glomerular filtration of endogenous polypeptides. Neutrophil gelatinase-associated lipocalin (NGAL) and soluble tumour necrosis factor (TNF) receptor 1 (sTNFr1) were measured as markers of neutrophil degranulation and monocyte activation, respectively. None of them showed any correlation with PR3, indicating increased circulating PR3 levels being independent of leucocyte activation. ANCA level and ANCA specificity had no significant influence. Remaining possible explanations for the increased circulating levels include selective leakage from granules, defects in the liver or reticulo-endothelial uptake of PR3/SERPIN complexes or factors leading to increased PR3 production. The latter possibility was supported by the results from Dr J. J. Yang *et al.* earlier this year, reporting up-regulation of the PR3 gene in leucocytes [9].

The monocytes play a central part in the scheme of inflammation and constitute a relatively homogeneous cell population with a versatile transcription apparatus. We chose to study PR3 expression in monocytes in order to investigate the possibility of up-regulated PR3 gene transcription. Peripheral blood monocytes were isolated from patients with AASV in different stages of disease activity, healthy blood donors (HBD) and patients suffering from systemic lupus erythematosus (SLE). As we found markedly increased levels among AASV patients we also studied other granular proteins such as human leucocyte elastase (HLE) and myeloperoxidase (MPO). To explore the possibility that the PR3 up-regulation was an effect of inflammation in general we also measured IL-8 mRNA and performed *in vitro* stimulation experiments using the monocyte like cell lines U937 and THP-1 [10] as well as human peripheral blood monocytes (PBMC) from healthy controls.

Methods

Patients

Twenty-two patients with AASV, according to the Chapel Hill Consensus Conference definitions, none of which were on dialysis or suffered from any bacterial or viral infections

or cancer, were consecutively included in this study (Table 1). Based on clinical observations performed by their regular physicians at the Department of Nephrology, Lund University Hospital, their status at the time of sampling was classified either as remission, chronic smouldering activity or relapse. Clinical status, BVAS, relapse tendency as well as the development of any severe organ damage due to vasculitic complications were registered. The clinical evaluation was conducted without access to the results of our analyses. The patients were grouped according to ANCA specificity (PR3 or MPO). Nine patients were MPO-positive and 12 patients were PR3-positive. Patient 22 had a diagnosis of Wegener's granulomatosis (restricted to the upper airways), but had never shown any positive results in ANCA analysis and is thus classified as 'seronegative'.

Our control groups comprised 15 HBD and 18 patients suffering from SLE. The majority of the patients with SLE were on corticosteroid therapy; four patients had doses of >15 mg/day (Table 2). The disease activity of the SLE patients is presented in Table 2 (SLEDAI: SLE disease activity index).

The studies were performed after approval from the Ethical committee at Lund University and written informed consent of the patients.

Blood samples

Venous blood, 48 ml, from each subject was obtained in ethylene diamine tetra-acetic acid (EDTA) tubes. The blood was put on ice and 32 ml was transferred immediately for monocyte purification. The remaining 16 ml were centrifuged within 1 h; plasma was aspirated carefully and stored at -20°C until assayed.

Enzyme-linked immunosorbent assay (ELISA)

PR3

ELISA was performed using monoclonal antibodies as capture antibodies, as described earlier [8].

MPO

A microtitre plate (Nunc immunoplate) was coated overnight with 100 µl/well of a monoclonal anti-MPO antibody, 2B11 [11], 1 µg/ml in coating buffer (0.01 M Na₂CO₃, 0.04 M NaHCO₃, 0.02% NaN₃, pH 9.5–9.7) at 4°C. The plate was blocked with coating buffer, containing 2% bovine serum albumin (BSA) for 30 min in room temperature and washed with 0.9% NaCl, 0.05% Tween 20 three times. All subsequent incubations were performed in 100 µl volumes at room temperature on a rocking table and followed by washing three times. Plasma samples diluted to 1/50, 1/100 and 1/200 in sample buffer [phosphate buffered saline (PBS) 7.3–7.4, 0.05% Tween 20, 0.2% BSA] were added and

Table 1. Patients with antineutrophil cytoplasmic antibodies (ANCA)-associated small vessel vasculitis.

Patient	Age	Sex	Status	Spec.	Titre	BVAS	CRP	WBC	Immunosuppressive treatment at the day of sampling
1	70	F	G	MPO	580	2	18	7.6	MTX12.5; P 2.5
2	55	M	R	MPO	>320	10	<5	8.4	CP 150; P 80
3	60	M	R	MPO	>320	24	13	6.9	None
4	62	M	R	MPO	184	13	74	7.3	None
5	52	F	R	MPO	147	15	<5	5.8	None
6	83	F	Q	MPO	41	0	<5	5.2	AZ 100; P 7.5
7	84	F	Q	MPO	37	0	12	4.6	P 5
8	52	F	R	MPO	25	8	<5	8.0	CP 150; P 60
9	68	F	Q	MPO	0	0	<5	7.6	MMF 500; P 7.5
10	67	F	Q	PR3	85	0	<5	4.7	AZA 100
11	59	F	Q	PR3	74	1	<5	6.1	CyA 150; P 2.5
12	68	F	G	PR3	37	2	9	5.9	MTX 12.5; P 10
13	51	F	Q	PR3	23	0	13	7.3	CP 75; P 7.5
14	26	M	G	PR3	17	3	<10	3.6	MTX 22.5; P 10
15	48	M	Q	PR3	0	0	<5	5.8	CP 100; P 7.5
16	68	M	Q	PR3	0	0	<9	6.0	AZA 100; P 7
17	45	M	Q	PR3	0	1	11	8.7	MTX 20; P 10
18	57	M	Q	PR3	0	0	<5	8.6	CP 125; P 20
19	74	M	Q	PR3	0	0	6	6.3	MTX 10; P 5
20	65	M	G	PR3	n.d.	3	16	7.9	AZ 100; P 10
21	61	M	G	PR3	n.d.	3	10	6.4	CyA 200; MMF 1000
22	28	M	Q	Seroneg	n.d.	0	<5	5.9	None

M = male; F = female. G = grumbling activity; Q = quiescent disease/remission; R = relapse; Spec = ANCA specificity; MPO = antibodies to myeloperoxidase (MPO-ANCA) at diagnosis; PR3 = antibodies to proteinase 3 (PR3-ANCA) at diagnosis; titre = results of ANCA determinations at the day of sampling [enzyme-linked immunosorbent assay (ELISA) units]; n.d. = not done; CRP = C-reactive protein (mg/l); WBC = white blood cell count ($\times 10^9/l$); MTX = methotrexate mg/week; P = prednisolone mg/day; CP = cyclophosphamide mg/day; AZ = azathioprine mg/day; CyA = cyclosporin A mg/day; MMF = mycophenolatemophetil mg/day.

Table 2. Control group, patients with systemic lupus erythematosus (SLE).

Patient	Age	Sex	SLEDAI	Immunosuppressive treatment at the day of sampling	PR3 expression by real time PCR
1	25	F	4	P 30; CP, pulse therapy	266
2	60	F	4	P 6	22
3	28	F	0	P 5; AZ 50	0.4
4	59	F	5	AZ 25	0.1
5	51	F	2	P 5; AZ 100	0.5
6	35	F	2	P 7.5; AZ 150	1.8
7	30	F	3	P 10; AZ 50	1.0
8	59	F	0	None	0.2
9	28	F	0	None	0.2
10	51	F	0	P 8.75	2.8
11	27	M	9	P 30; AZ 150	482
12	42	F	0	P 5	0.9
13	59	F	2	None	0.7
14	21	F	1	P 10; MMF 500	2.4
15	25	M	8	P 20	46
16	57	F	10	P 20; MMF 500	216
17	60	F	1	P 10; AZ 50	0.9
18	24	F	0	P 2.5; MMF 2000	3.4

M = male; F = female. SLEDAI = SLE disease activity index. P = prednisolone mg/day; CP = cyclophosphamide mg/day; AZ = azathioprine mg/day; MMF = mycophenolatemophetil mg/day; PCR = polymerase chain reaction; PR3 = proteinase 3. The real time data are corrected according to the $2^{-\Delta\Delta CT}$ formula and then expressed in relation to the median value of the healthy controls.

the plate incubated for 1 h. After washing MPO was detected by 1-h incubation with rabbit anti-MPO (Dako, Glostrup, Denmark) diluted to 1/2000 in sample buffer. Washing was followed by addition of the conjugate (alkaline phosphatase-labelled swine anti-rabbit IgG, Dako), diluted to 1/1000 in sample buffer and 1-h incubation. P-nitrophenyl-phosphate disodium (Sigma-Aldrich Corp., St Louis, MO, USA) 1 mg/ml in substrate buffer (1.0 M diethanolamine, pH 9.8, 0.5 mM MgCl₂, 0.02 NaN₃) was used as substrate and optical densities were read at 405 nm. A standard curve was produced by incubation of a twofold dilution series of MPO (Wieslab AB, Lund, Sweden), starting with 10 ng/ml.

IL-6, IL-8

A quantitative sandwich enzyme immunoassay from R&D Systems (Abingdon, UK), where a monoclonal antibody specific for either IL-6 or IL-8 had been precoated onto a microplate, was used.

Cystatin C, CRP, WBC

The Clinical Chemical Laboratory at Lund University Hospital, Lund, Sweden, performed analyses on a Hitachi 917 Pluto. Kits from Roche Diagnostics (Basel, Switzerland) and Dako were used.

ANCA

Wieslab AB, Lund, Sweden, performed analyses of PR3-ANCA and MPO-ANCA using routine methods [12].

Cell separation

Peripheral blood monocytes were isolated by means of a monocyte isolation technique based on the OptiPrep density-gradient medium (Axis-Shield PoC AS, Oslo, Norway) [13]. The method was carried out at 4°C, during sterile conditions, using sterile solutions. Briefly, OptiPrep working solution (WS) was added to whole blood. A centrifugation gradient was created by mixing WS and solution B [Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% serum; Invitrogen, Carlsbad, CA, USA]. Five ml blood was pipetted into a 15 ml test tube, after which 5 ml gradient was added carefully and finally 0.5 ml solution B on top (in order to avoid banding of the cells at a liquid/air interface). During the following centrifugation (700 g, 30 min, 4°C, no brake during deceleration) the monocytes float to the top of the gradient layer. After collection, the cells were gently diluted with 2 vol solution B, harvested by centrifugation and resuspended in solution B. Twenty-five µl cell suspension was then mixed with Türk's solution (methyl-violet) and counted in a Bürker chamber. A cell smear from each sample was also stained

with May-Grünwald for differential counting. The monocyte purity was 85–95%, with single contaminating lymphocytes.

RNA extraction

Total RNA was extracted with RNeasy Mini kit (Qiagen, VWR International, West Chester, PA, USA) using the supplied protocol. High purity and good integrity were determined in two ways: first by optical density, 260/280 nm spectrophotometric ratios and then by the Agilent 2100 Bioanalyser, using the RNA 6000 Nano Assay reagent kit (Agilent Technologies, Palo Alto, CA, USA). After adding a gel-dye mix together with the RNA sample (25–500 ng) to the RNA 6000 Nano Chip channel system, the bioanalyser uses electrophoretic and electro-osmotic forces to drive fluids through capillaries to produce a virtual gel image and an electropherogram. In the electropherogram RNA of good quality shows up with clear 18 S and 28 S rRNA peaks and a flat baseline, whereas in the gel you see the corresponding sharp bands – the larger ribosomal band being more intense. Only RNA samples that met these criteria were accepted for further analyses.

Quantitative PCR assay

Total RNA was transcribed into cDNA, using the *TaqMan* Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations. In short, random hexamers were used as template and put into the mastermix together with MultiScribe reverse transcriptase, RNase inhibitor, dNTPs, 5.5 mM MgCl₂ and reverse transcription buffer. Five hundred ng total RNA was added to each 50-µl reaction and put into the thermocycler, set at 25°/10 min, 48°/30 min and 95°/5 min. For determination of gene expression, quantitative PCR assays were performed on an ABI PRISM 7000 Sequence Detector (Applied Biosystems) with *TaqMan* Universal Master Mix UNG, using the standard conditions determined by the company. After UNG incubation for 2 min at 50° and *AmpliTaq* Gold activation for 10 min at 95°, 40 cycles were run with denaturing temperature 95° (15 s) and annealing/extension temperature 60° (1 min). Assay on Demand, a unique combination of forward and reverse primers and fluorescent MGB-probes designed by the company, was used for each target gene. β-actin expression levels were used for normalization. cDNA corresponding to 10 ng RNA was used per 25 µl reaction and each reaction was performed in triplicate. The level of expression was calculated based upon the PCR cycle number (Ct) at which the exponential growth in fluorescence from the probe passes a certain threshold value. Relative expression was determined by the difference in the Ct values for the target genes after normalization to RNA input level, using β-actin Ct values. Relative quantification was determined by standard $2^{(-\Delta\Delta C_t)}$.

calculations [14]. Data are presented in relation to the median value of the HBD, set as 1.

In vitro stimulation assay

Stimulation of cell-lines U937/THP1 cells

The monocyte-like cell lines THP-1 and U937-4, a subclone of U937, were stimulated with cytokines and lipopolysaccharide (LPS) for different time periods [10]. The cells were cultured in suspension in RPMI-1640 with 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA, USA) and exposed to interferon (IFN)- γ or IFN- α 100 U/ml (Boehringer, Ingelheim, Germany), LPS 10 μ g/ml (Sigma-Aldrich Corp.), TNF- α 20 ng/ml (ICN Biomedicals, Aurora, Ohio, USA) and LPS 10 μ g/ml plus TNF- α 20 ng/ml. The cells were harvested after stimulation and incubation for 6 and 20 h, respectively. Total RNA was extracted using the Trizol LS reagent (Invitrogen). Real time data are presented in relation to cells that have been incubated for 6 *versus* 20 h, without stimuli, set as 1.

Stimulation of human PBMC from healthy controls

The cells were purified using Optiprep as described above and 10^6 cells/well were cultured in RPMI-1640 with 10% FCS (Invitrogen), 10 ng/ml IL-4 and 20 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) for 1 h. The cells were then exposed to IFN- γ 100 U/ml (Boehringer), LPS 10 μ g/ml (Sigma-Aldrich Corp.) and TNF- α 10 ng/ml (ICN Biomedicals), respectively, and harvested after 6 h. Total RNA was extracted using the RNeasy Mini kit. Real time data are expressed in relation to non-stimulated cells, set as 1.

Statistical analysis

All statistics were performed in STATVIEW 5.01. Due to non-normally distributed parameters, the non-parametric Spearman's rank correlation test was used for correlation analysis in order to reduce the impact of outliers. Analysis of variance was performed using the non-parametric Kruskal–Wallis test and Mann–Whitney *U*-test.

Results

PR3 and MPO levels in plasma

Compared to HBD, we found increased circulating levels of the PR3 protein in patients with AASV, regardless of ANCA specificity ($P < 0.0001$) (Table 3). MPO levels were also found to be higher in the patients with AASV compared to HBD ($P < 0.05$). The SLE patients had higher levels of PR3 in plasma than the HBD (non-significant), but not to the same extent as in AASV, whereas MPO levels were similar to AASV. IL-6 and IL-8 were measured in plasma as inflammatory markers, showing similar inflammatory activity in the two disease groups.

mRNA levels in monocytes

Compared to both HBD ($P < 0.0001$) and SLE patients ($P = 0.01$), we found a 10-fold increase of PR3 expression in monocytes from patients with ANCA-associated vasculitis (Table 4). As shown in Fig. 1, all but two of the ANCA-positive patients had a PR3 expression greater than the maximum HBD expression. Four of our patients had no ongoing immunosuppressive treatment, whereas the others had different combinations of corticosteroids and other immunosuppressants. Four of the SLE patients had high PR3 expression (46–482). These patients all had relatively high doses of corticosteroids, at similar levels to AASV patients 2 and 5, and they were all active in their disease (Tables 1, 2). The other patients had low doses of corticosteroids. A fourfold increase of HLE expression was seen among patients with AASV and a threefold increase among SLE patients ($P < 0.05$ when compared to HBD). MPO and bactericidal permeability-increasing protein (BPI) expression did not differ from that in HBD for patients with AASV. IL-6 and IL-8 were measured in plasma as inflammatory markers (Table 3). On the RNA-level, IL-6 expression was very low, and data were therefore unreliable and not shown. This observation indirectly contradicts an unspecific activation of the cells during the purification process. IL-8 expression was increased in the SLE group but not in the AASV group, indicating a difference in inflammatory mobilization, as shown in Table 4.

Table 3. Proteinase 3 (PR3) and myeloperoxidase (MPO) protein levels in plasma.

	PR3 (μ g/l)	MPO (μ g/l)	IL-6 (ng/l)	IL-8 (ng/l)
AASV	560 (110–3940)	74 (14–120)	3.2 (1.6–8.6)	8.3 (2.4–18.9)
MPO-pos	560 (380–1770)	68 (14–103)	3.9 (1.8–7.8)	7.2 (2.4–18.9)
PR3-pos	570 (110–3940)	74 (45–120)	3.1 (1.6–8.6)	9.1 (7.7–17.1)
HBD	350 (110–580)	15 (12–18)	1.0 (0.3–6.1)	4.7 (2.4–8.8)
SLE	435 (138–959)	69 (49–90)	3.0 (1.2–8.7)	5.7 (3.7–12.0)

AASV = all patients with ANCA-associated vasculitis; MPO-pos = patients with MPO-ANCA at diagnosis; PR3-pos = patients with PR3-ANCA at diagnosis; HBD = healthy blood donors; SLE = disease controls with systemic lupus erythematosus; IL: interleukin. Data are presented as median (range).

Table 4. Monocyte expression profile, relative mRNA levels.

	PR3	HLE	MPO	BPI	IL-8
AASV, <i>n</i> = 22	9.6 (1.8–680)	4.0 (0.6–52)	2.1 (0.4–14)	1.4 (0.3–12)	0.9 (0.03–13)
MPO-pos, <i>n</i> = 9	15.4 (5.4–70)	5.7 (0.6–12)	2.1 (0.8–8.1)	1.4 (0.7–12)	1.3 (0.2–13)
PR3-pos, <i>n</i> = 12	8.1 (1.8–680)	2.5 (0.9–52)	1.6 (0.4–14)	1.5 (0.3–7.6)	0.3 (0.03–6.4)
ANCA-neg, <i>n</i> = 1	0.8	0.6	0.3	0.3	2.0
HBD, <i>n</i> = 15	1.0 (0.1–2.8)	1.0 (0.2–4.8)	1.0 (0.4–8.1)	1.0 (0.3–23)	1.0 (0–42)
SLE, <i>n</i> = 18	3.1 (0.1–480)	1.4 (0.4–540)	5.7 (1.0–150)	1.2 (0.3–34)	5.2 (0.2–33)

AASV = all patients with antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis; MPO-pos = patients with myeloperoxidase (MPO)-ANCA at diagnosis; PR3-pos = patients with proteinase 3 (PR3)-ANCA at diagnosis; HBD = healthy blood donors; SLE = disease controls with systemic lupus erythematosus. The real time data are corrected according to the $2^{-\Delta\Delta CT}$ formula and then expressed in relation to the median value of the healthy controls. Results are shown as median (range).

In patients with AASV, plasma levels of PR3 tended to reach higher levels in patients with greater expression, but the correlation was not statistically significant (Fig. 2). No significant correlation was seen between plasma levels of CRP, IL-6 or IL-8 and PR3 expression. The correlation coefficients were -0.01 , 0 and 0.4 , respectively. PR3-ANCA titres showed negative correlation with PR3 expression ($p = -0.7$, $P < 0.05$), whereas the opposite was seen with white blood cell counts (WBC, $p = 0.6$, $P < 0.05$). There was no correlation between PR3 expression and the monocyte counts.

In the ANCA patients PR3 expression covaried to some extent with HLE, and to a minor extent with MPO and BPI. The SLE patients, however, exhibited an apparent covariation between all four measured granular proteins (Table 5).

In vitro stimulation of monocyte-like cell lines and PBMC

Stimulation of the monocyte like cell lines U937 and THP1 and human PBMC from healthy controls with various cytotok-

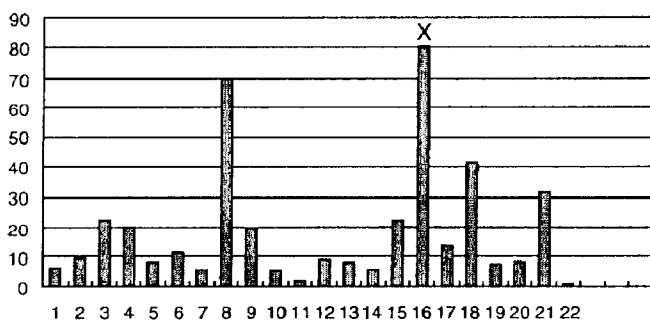


Fig. 1. Monocyte proteinase 3 (PR3) mRNA levels among 22 patients with antineutrophil cytoplasmic antibodies (ANCA)-associated small vessel vasculitis. Patient enumeration is the same as in Table 1. Patients 1–9 are myeloperoxidase (MPO)-ANCA positive, 10–21 are PR3-ANCA-positive and patient 22 is seronegative. Levels are normalized using β -actin as housekeeping gene and the median value of our 15 healthy controls was set as 1.0 (range 0.1–2.8). The difference between ANCA-positive patients and healthy controls is highly significant, while the difference between MPO-ANCA and PR3-ANCA is not significant. X = outlier reaching 680.

ines and LPS resulted in activation, as a substantial up-regulation of the IL-6 and IL-8 mRNA levels was seen. This, however, was not accompanied by any increase in the mRNA for the granule constituents PR3 or HLE, as shown in Tables 6, 7.

Table 5. Covariation of the relative mRNA levels of some monocyte genes.

	IL-8	BPI	MPO	HLE
PR3, AASV	0.2 n.s.	0.5*	0.6*	0.7**
PR3, SLE	–0.4 n.s.	0.8**	0.8**	0.9**
HLE, AASV	0.5*	0.7*	0.7*	
MPO, AASV	0.4*	0.6*		
BPI, AASV	0.5*			

AASV = antineutrophil cytoplasmic antibodies (ANCA)-associated systemic vasculitis; SLE, systemic lupus erythematosus; MPO = myeloperoxidase; HLE = human leucocyte elastase; PR3 = proteinase 3; IL = interleukin. Correlations are expressed as Rho; * $P < 0.05$; ** $P < 0.001$; n.s. $P > 0.05$.

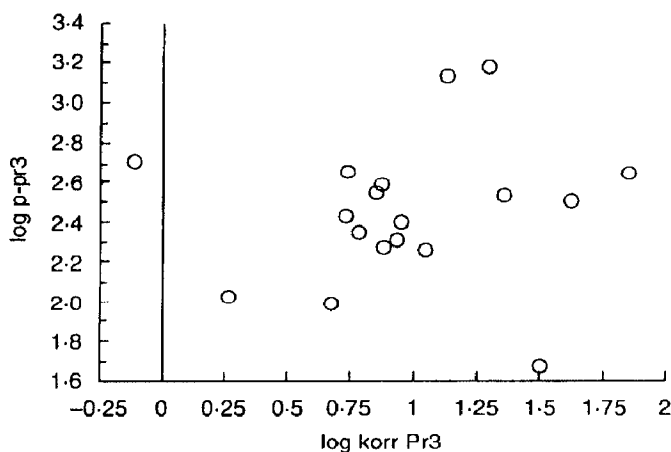


Fig. 2. Correlation between plasma proteinase 3 (PR3) concentrations and monocyte PR3 mRNA levels among 18 of 22 patients with antineutrophil cytoplasmic antibodies (ANCA)-associated small vessel vasculitis. Plasma levels are measured in $\mu\text{g/l}$ using enzyme-linked immunosorbent assay (ELISA). mRNA levels are normalized using β -actin as housekeeping gene and the median value of the healthy controls is set to 1. The correlation coefficient is 0.3 (not significant) when using the Spearman's rank correlation test.

Table 6. Cytokine stimulation of PR3, HLE and IL-8 expression in THP1 and U937 cell lines.

	PR3	HLE	IL-8	IL-6
THP1, 6 h				
unstimulated	1	1	1	1
IFN- γ	1.5	1.3	–	–
TNF- α	0.8	0.6	10.5	3.0
LPS + TNF- α	0.8	0.7	4.0	2.8
THP1, 21 h				
unstimulated	1	1	–	–
IFN- γ	0.5	0.5	–	–
TNF- α	0.3	0.3	–	–
LPS	0.6	0.8	19.3*	–
IFN- α	0.3	–	–	–
U937, 6 h				
unstimulated	1	–	–	1
IFN- γ	0.7	–	–	24.3
TNF- α	0.8	–	–	4.7
LPS	0.9	–	–	2.5
U937, 21 h				
unstimulated	1	–	–	–
TNF- α	0.1	–	–	–
LPS	0.3	–	–	–
IFN- α	0.3	–	–	–
Mono, 21 h				
unstimulated	1	–	–	–
IFN- α	1.1	–	–	–

The cells were incubated for 6 and 21 h, respectively. The real time reverse transcription-polymerase chain reaction (RT-PCR) data are corrected according to the $2^{-\Delta\Delta C_T}$ formula and expressed in relation to unstimulated cells, incubated for the same length of time. *Unstimulated THP1 incubated 6 h set as 1 in this experiment. – = not done; IFN- γ = interferon gamma 100 U/ml; TNF- α = tumour necrosis factor alpha 20 ng/ml; LPS = lipopolysaccharide 10 μ g/ml. Mono = human monocytes; IFN- α = interferon alpha, 1000 U/ml.

Discussion

This study demonstrates a strong relative increase in mRNA levels for PR3 in monocytes from patients with AASV compared to both HBD and disease controls. The median result for PR3 mRNA was a 10-fold increase, and the separation between HBD and AASV was remarkable. The correlation between plasma PR3 concentration and monocyte PR3 mRNA was positive but weak, and failed to reach statistical significance when using a non-parametric test. This is, however, not surprising considering that there are several steps between mRNA levels and circulating protein. High levels of PR3 mRNA in circulating monocytes do not necessarily mean an increased total amount of PR3. PR3 is also produced by neutrophils, which are more abundant, and our data concern normalized RNA and thus indicate PR3 production per cell rather than the total production in the body. Furthermore, autoantibodies against PR3 might influence the half-life of PR3 in the circulation and/or the measurement of PR3 in plasma. In the present study higher levels of PR3-ANCA were associated with lower levels of circulating

PR3 (data not shown). There was also a negative correlation between ANCA levels and PR3 mRNA in the monocytes, which argue against a direct causative effect of the autoantibodies on the PR3 mRNA production. An *in vitro* study by Yang and coworkers did not find PR3 among genes up-regulated in neutrophils treated with ANCA-IgG [15].

PR3 is normally transcribed during myelopoiesis and is supposed to be turned off in mature leucocytes [16]. Constitutive expression of PR3 is a feature of many haematopoietic malignancies where a differentiation block have prevented the maturation and kept the cells in a proliferative state [17]. An alternative name for PR3 is myeloblastin, which was first described as a substance that maintained the proliferative capacity of myeloblasts. Anti-sense treatment blocking PR3 transcription led to growth arrest and differentiation of promyelocytic leukaemia cells [18].

Recently there have been several reports showing that PR3 production can occur in more mature cells. Zhou *et al.* have published a study showing *de novo* synthesis of PR3 by circulating mononuclear cells that were cultured and stimulated with TNF- α . This study was performed using mononuclear cells from healthy donors and the percentage of monocytes/lymphocytes was 40/60, leaving a potential lymphocyte influence on the results [19]. Another study, by Just *et al.*, showed an up-regulation of PR3 mRNA expression in circulating monocytes, but not in neutrophils, in cystic fibrosis patients, correlating with pulmonary exacerbation [20]. Brockman *et al.* found increased PR3 expression in macrophages at inflammatory sites in lung tissue from patients with WG [21]. Earlier this year Yang *et al.* demonstrated increased PR3 transcription in circulating leucocytes from patients with AASV compared with HBD and SLE patients, correlating with disease activity [9]. These reports indicate that our results might be a result of cytokine action caused by general inflammation. There are several findings in our study that argue against this notion: (1) there was no correlation between circulating IL-6, IL-8 or CRP-levels and PR3 expression; (2) there was no significant IL-6

Table 7. Cytokine stimulation of PR3, human leucocyte elastase (HLE) and interleukin expression in healthy peripheral blood monocytes.

	PR3	HLE	IL-8	IL-6
Culture medium	0.4	0.3	2.3×10^4	0.4×10^4
LPS	0.2	0.2	10×10^4	1.2×10^4
TNF- α	0.3	0.2	3.2×10^4	0.2×10^4
IFN- γ	0.2	0.1	3.5×10^4	0.4×10^4

The cells were incubated with stimuli for six hours. Data are presented as mean values of peripheral blood mononuclear cells (PBMC) from three separate donors, in relation to the corresponding freshly drawn, non-stimulated samples. Culture medium = RPMI-1640 with 10% fetal calf serum (FCS), 10 ng/ml interleukin (IL)-4 and 20 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF); LPS = lipopolysaccharide, 10 μ g/ml; TNF- α = tumour necrosis factor alpha, 10 μ g/ml; IFN- γ = interferon gamma, 100 μ g/ml; PR3 = proteinase 3.

or IL-8 up-regulation on the mRNA level in the monocytes; (3) there was no general up-regulation of granular proteins; and (4) *in vitro* stimulation of monocyte-like cell lines and healthy PBMC with TNF- α , IFN- γ or LPS for 6 or 20 h did not result in any increase of PR3 production, whereas the IL-8 expression was highly up-regulated.

In addition to the increased PR3 levels we also found a minor increase in HLE mRNA levels when compared to HBD, but we found no significant alteration of MPO or BPI. These results differ from those of Dr Yang, which indicated a more general up-regulation of granular proteins [9]. What we did see was a strong covariation of the transcription of the granular enzymes in the SLE patients, thus there were both quantitative and qualitative differences between SLE and AASV monocyte gene transcription in the present study. There are reports that granulocyte colony stimulating factor (G-CSF) can up-regulate PR3 transcription [22]. Indeed we found a positive correlation between total white blood cell count and monocyte PR3 mRNA. The G-CSF was shown to stimulate through the G-CSF receptor and the transcription factor PU.1. Because PU.1 response elements are present in the vast majority of promoters for granule constituents, high G-CSF levels are unlikely to be responsible for the qualitative difference between AASV and SLE patients.

Another concern is that of immunosuppressive drugs. This was a major reason for choosing SLE patients as disease controls. Fifteen of 22 patients with AASV had low doses of corticosteroids and three patients had high doses (exceeding 15 mg per day). The latter three had high PR3 expression, but otherwise there was no correlation with the corticosteroid dose. Four of the SLE patients also had high doses of corticosteroids and they exhibited high expression of all measured granular mRNAs, indicating that above a certain dose corticosteroids could influence PR3 expression. Three of our AASV patients were treatment naive, but still demonstrated considerably raised PR3 expression.

A skewed distribution of a polymorphism in the promoter region of the PR3 gene, involving a transcription factor binding site, have recently been described [23]. Because the difference in allele frequency between patients and controls was relatively small, it is very unlikely that this polymorphism could explain our present findings. This notion has been further supported by the findings in our recently published study, showing that the -564 A/G polymorphism does not increase PR3 promoter activity [24].

If the increased PR3 levels in AASV are not caused by inflammation, medication, autoantibodies or germline genetic variants, what could then be the cause? Our study, as well as the work of Dr Yang *et al.* [9], has demonstrated up-regulated PR3 gene transcription in AASV. This would imply an increased PR3 production. A highly speculative hypothesis would be that a somatic mutation occurs in a stem cell leading to very late maturation block with increased PR3 production as a major feature. This is a bold hypothesis in need of extensive work in order to be thoroughly evaluated.

In conclusion, circulating monocytes from patients with systemic vasculitis display up-regulated transcription of the PR3 gene, implying increased PR3 production and a potential source of the increased circulating levels described earlier [8]. Considering the great toxic potential of PR3, this may be of importance for the development of vasculitis. The origin of this potentially increased production remains obscure; however, our results do not favour an influence of cytokines, ANCA or immunosuppressive medication. All these aspects, however, require further study.

Acknowledgements

This study was supported by grants from the Swedish Scientific Council (project 71X-09487 and 74X-13489), the foundations of the Royal Physiographic Society and Thelma Zoega, the Foundation for Strategic Research, Riksförbundet för Njursjuka, Sweden. We wish to thank Nermina Jaganjac, Åsa Pettersson (Department of Nephrology) and Carina Strand (Department of Oncology) for skilful laboratory assistance.

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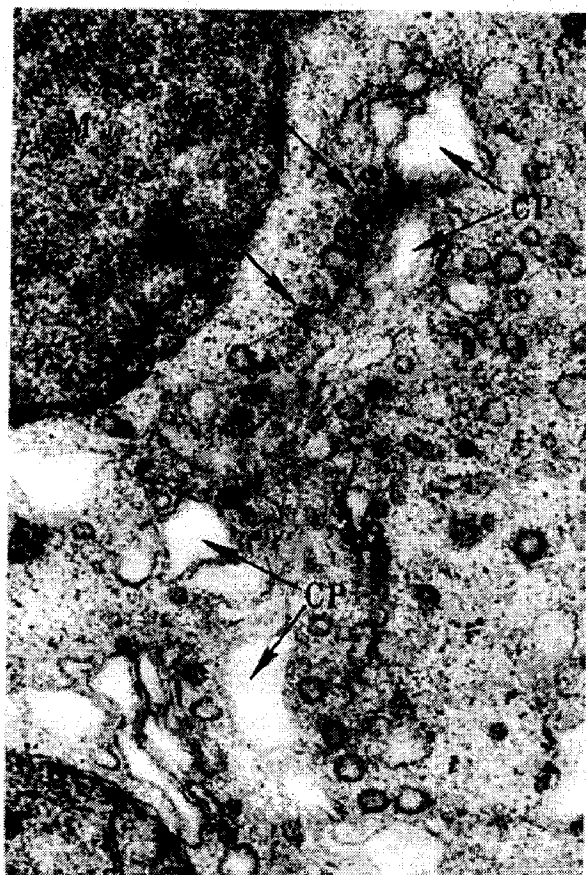


Figure 1-41. Electron micrograph of a Golgi apparatus from a normal human proximal tubule. Small vesicles (arrows) consistent with the appearance of primary or virgin lysosomes are seen budding from the larger cisternal profiles (CP). (M = mitochondrion) (Magnification $\times 32,900$) (From Tisher, C. C., et al.: *Lab. Invest.* 15:1357, 1966.)

proximal tubule cells, especially in the pars convoluta, is an extensive system of microtubules.

An important function of the proximal convoluted tubule and, to a lesser extent, the pars recta is the reabsorption and degradation of various macromolecules including albumin and low molecular weight plasma proteins from the glomerular filtrate. The initial stage of the process, termed endocytosis or pinocytosis, involves the apical tubules, vesicles, and vacuoles. Maunsbach,¹²⁷ studying the uptake of the electron-dense protein, ferritin, and homologous albumin¹⁶⁴ placed directly into the lumen of the rat proximal tubule, demonstrated ultrastructurally that small quantities of the proteins become located in invaginations of the luminal cell membrane which then pinch off to form apical vesicles. The apical vesicles fuse with one another to form apical vacuoles or fuse with preexisting apical vacuoles. The apical vacuoles move toward the center of the cell where they fuse with lysosomes containing acid hydrolases that are necessary for the digestion of the ingested material. It is believed currently that at least one source of acid hydrolases is the Golgi vesicle that pinches off from the Golgi cisternae.

Recent micropuncture studies by Christensen et al.,¹⁶⁵ studies in the isolated perfused kidney by Sumpio and Maack,¹⁶⁶ and studies by Park and Maack¹⁶⁷ using the isolated perfused tubule have provided evidence that the

absorption of protein by the proximal tubule is a selective process determined by the net charge of the protein and the charge distribution on the molecule, the size and configuration of the protein molecule, and possibly the presence of preferential endocytic sites for certain proteins.

Numerous experimental light microscopic and electron microscopic studies have demonstrated the ability of the proximal tubule to reabsorb substances such as hemoglobin, horseradish peroxidase, mannitol, sucrose, dextran, myoglobin, lactoperoxidase, and lysozyme. The interested reader is referred to reference 111 for a more detailed discussion of the subject. In addition, amphiphilic cationic drugs such as chloroquine and tricyclic antidepressants,¹⁶⁸ aminoglycoside antibiotics^{169, 170} and certain heavy metals¹⁷¹ accumulate in lysosomes in the proximal tubule giving rise to structural as well as functional changes in the lysosomal system.

Recent studies have demonstrated that lysosomal digestion of absorbed proteins can be influenced by such factors as aging and heavy metal intoxication. For instance, Christensen and Madsen observed a significant decrease in the digestion of ¹²⁵I-labeled lysozyme by proximal tubule lysosomes of aging rats.¹¹⁸ Madsen and Christensen also demonstrated a decrease in the catabolism of the low-molecular-weight proteins, lysozyme and cytochrome c, by lysosomes of the proximal tubule during chronic mercury intoxication.¹⁷²

In addition to its role in heterophagy, the lysosomal system is involved in autophagocytosis or autophagy in the proximal tubule in normal as well as pathological conditions.¹⁷³ By this process small amounts of cytoplasm and cell organelles such as mitochondria, microbodies, and ribosomes that are no longer essential or of functional value to the cell are first segregated within profiles of smooth-surfaced endoplasmic reticulum. The open ends of the enveloping smooth-surfaced endoplasmic reticulum fuse to form a double-walled sac and later a single-walled organelle, termed an autophagic vacuole; with the addition of acid hydrolases through fusion with a primary lysosome, this organelle becomes an autolysosome. Finally, through a process termed exocytosis, residues of undigested material enclosed within lysosomal structures, termed telolysosomes or residual bodies, leave the cell at the luminal border (Fig. 1-39). The cellular processes of heterophagy, autophagy, and exocytosis are summarized diagrammatically in Figure 1-42.

PARS RECTA. As noted in an earlier section, the pars recta of the proximal tubule includes the latter portion of the S₂ segment and all of S₃ to its transition into the thin descending limb of Henle. The epithelium of the pars recta is generally cuboidal; the apical cell surface is convex and is covered by a well-developed brush border composed of elongated microvilli. In the rat the microvilli of the more distal portion of the pars recta measure up to 4.0 μ m in length¹⁷⁴ (Fig. 1-26). In the rabbit¹¹⁴ and the human¹¹⁷ pars recta the microvilli are considerably shorter (Fig. 1-43). Electron microscopic studies demonstrate a virtual absence of lateral and basilar interdigitations between cells thereby decreasing the size of the extracellular space between the basement membrane and the tight junction (basal labyrinth) as one moves downward from the cortical to the medullary segment of the pars recta.^{111, 174} These findings coupled with a reduction in

invited review

Megalin and cubilin: synergistic endocytic receptors
in renal proximal tubule

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Christensen, Erik Ilsø, and Henrik Birn. Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. *Am J Physiol Renal Physiol* 280: F562–F573, 2001.—The multiligand, endocytic receptors megalin and cubilin are colocalized in the renal proximal tubule. They are heavily expressed in the apical endocytic apparatus. Megalin is a 600-kDa transmembrane protein belonging to the low-density lipoprotein-receptor family. The cytoplasmic tail contains three NPXY motifs that mediate the clustering in coated pits and are possibly involved in signaling functions. Cubilin, also known as the intestinal intrinsic factor-cobalamin receptor, is a 460-kDa receptor with no transmembrane domain and no known signal for endocytosis. Because the two receptors bind each other with high affinity and colocalize in several tissues, it is highly conceivable that megalin mediates internalization of cubilin and its ligands. Both receptors are important for normal tubular reabsorption of proteins, including albumin. Among the proteins normally filtered in the glomeruli, cubilin has been shown to bind albumin, immunoglobulin light chains, and apolipoprotein A-I. The variety of filtered ligands identified for megalin include vitamin-binding proteins, hormones, enzymes, apolipoprotein H, albumin, and β_2 - and α_1 -microglobulin. Loss of these proteins and vitamins in the urine of megalin-deficient mice illustrates the physiological importance of this receptor.

proteinuria; vitamin D; vitamin B₁₂; retinol; low-density lipoprotein-receptor family

MEGALIN IS A MULTILIGAND, endocytic receptor belonging to the low-density lipoprotein (LDL)-receptor family. It is heavily expressed in the renal proximal tubule. A long list of ligands for megalin has been identified establishing an important role for the tubular uptake of filtered proteins. Among the ligands are vitamin binding proteins and several hormones, suggesting an additional role of megalin in the metabolism and homeostasis of essential vitamins, including vitamin D, as well as calcium.

Cubilin, also known as the intestinal intrinsic factor-cobalamin receptor, is coexpressed with megalin in the renal proximal tubule. Although structurally very different from megalin, it has many similar features, being a multiligand, endocytic receptor sharing several

ligands with megalin and being important for the tubular reabsorption of proteins. In addition, megalin has been shown to bind cubilin and is most likely involved in the endocytosis of this receptor.

The present review will focus on megalin and cubilin in the kidney, their structural features, mutual interaction, potential signaling function, and their role in tubular protein reabsorption, vitamin metabolism, as well as calcium homeostasis.

HISTORY*Megalin*

Megalin was originally identified as the antigen in Heymann nephritis of rats. It was purified from rat kidney brush border and named gp330 on the basis of molecular weight as estimated by its mobility during gel electrophoresis (43). Megalin was localized in the apical endocytic pathway of renal proximal tubule as

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well as in the glomeruli (43). Later, it was identified in several other epithelia (for a recent review, see Ref. 16). Many proteins were identified as ligands, including receptor-associated protein (RAP), lipoproteins, enzymes, and enzyme inhibitors, suggesting the role of megalin as a scavenger endocytic receptor. In 1994, the protein was cloned by Saito et al. (81), showing it to be a 600-kDa glycoprotein and suggesting the name "megalin." In 1996, megalin-deficient mice was produced by gene targeting by Willnow et al. (92), adding significant new information about the important role of this receptor.

Cubilin

Cubilin was identified as the target of teratogenic antibodies produced by the injection of renal brush-border preparations into rabbits (78). It was named gp280 on the basis of the estimated molecular weight and localized to the apical endocytic apparatus of renal proximal tubule and the visceral epithelia of yolk sac (78). No ligands were identified until 1997, when gp280

was shown to be identical to the intestinal intrinsic factor-cobalamin receptor (84). Also, in 1997 RAP was shown to be a ligand for cubilin, followed by several other proteins. The receptor was cloned by Moestrup et al. (65) in 1998 and shown to be a 460-kDa glycoprotein with no apparent cytoplasmic domain, and the name "cubilin" was suggested on the basis of its structure dominated by complement subcomponents C1r/C1s, Uegf, and bone morphogenic protein-1 (CUB) domains.

STRUCTURE

Megalin

Megalin is an ~4,600-amino acid transmembrane protein (Fig. 1) with a large NH₂-terminal extracellular domain, a single transmembrane domain, and a short cytoplasmic tail (81). The protein belongs to the LDL-receptor family (76), sharing common features with the following mammalian receptors including the LDL receptor, the LDL-receptor-related protein (LRP), the very-low-density lipoprotein (VLDL) receptor, and

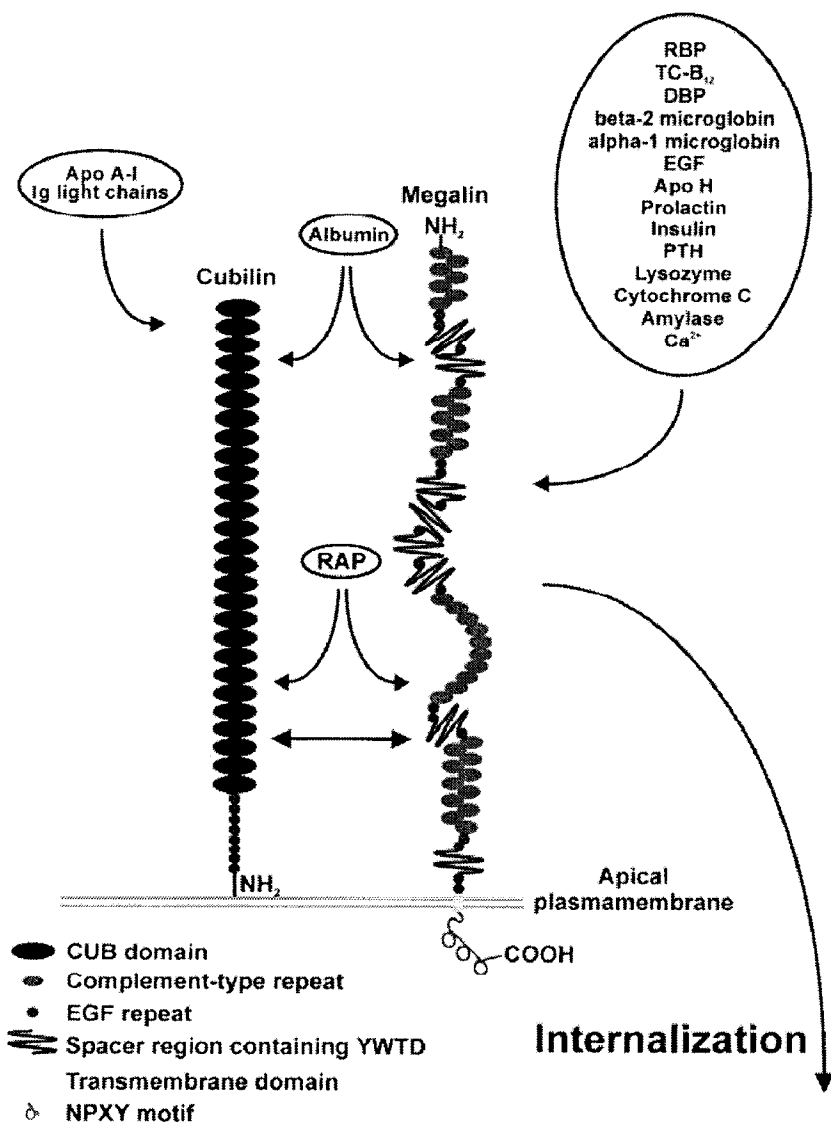


Fig. 1. Illustration of the structural organization and interaction between cubilin and megalin. The C1r/C1s, Uegf, and bone morphogenic protein-1 (CUB) domains in cubilin and the complement-type repeats in megalin represent the ligand binding regions of the 2 receptors. Cubilin is a peripheral membrane protein, whereas megalin is a transmembrane protein with 3 cytoplasmic NPXY motifs, directing the receptor into coated pits. A number of filtered ligands for each of the 2 receptors in the kidney proximal tubule are also depicted, in addition to albumin and receptor-associated protein (RAP), which bind to both receptors. Finally, the binding of cubilin to megalin and the megalin-mediated internalization of the ligand-receptor-receptor complex are indicated. Apo, apolipoprotein; RBP, retinol-binding protein; TC, transcobalamin; DBP, vitamin D-binding protein; EGF, epidermal growth factor; PTH, parathyroid hormone.

the apolipoprotein E (apo E) receptor-2 (reviewed in Ref. 29). Both rat (81) and human (37) megalin has been cloned, and the nonglycosylated molecular mass is estimated to 517 kDa (81). The cytoplasmic tail contains three NPXY motifs mediating the binding to adaptor proteins and the clustering into coated pits. In addition, this motif may serve signaling functions (75). Also, the cytoplasmic domain contains several Src homology 3 and one Src homology 2 recognition sites (37). The extracellular domain contains four cysteine-rich, complement-type repeats, probably constituting the ligand binding regions separated by epidermal growth factor (EGF) precursor homology domains containing YWTD repeats responsible for the pH-dependent release of ligands (24). The human megalin gene has been located to chromosome 2q24-q31 (46).

Cubilin

Cubilin is an ~3,600-amino acid protein with no transmembrane domain (Fig. 1). The complete DNA sequences of rat (65), human (52), and canine (96) cubilin have been identified, showing a nonglycosylated molecular mass of 400 kDa. It has little structural homology with other known endocytic receptors. The extracellular domain contains 27 CUB domains. The CUB domains most likely constitute the ligand binding domains, and the binding site for intrinsic factor-cobalamin has been located within CUB domains 5–8 whereas the binding site for RAP is located within CUB domains 13–14 (54). The CUB domains are preceded by a stretch of 110 amino acids followed by 8 EGF-type repeats. The initial amino acid stretch contains a furin cleavage site, which may indicate proteolytic processing in the *trans*-Golgi network (52). The NH₂-terminal region seems essential to membrane anchoring of the protein. This segment contains an amphipathic helix structure with some similarity to the lipid binding regions of apolipoproteins, which may contribute to the anchoring of the receptor in the membrane (54). The human cubilin gene has been located to chromosome 10p12.33-p13 (52).

EXPRESSION AND SYNTHESIS

Megalyn

Megalyn is expressed in many epithelial cells (49, 99; recently reviewed in Ref. 16), in particular absorptive epithelia facing transcellular fluids, such as the renal proximal tubule, the glomerular podocytes, the choroid plexus, ependymal cells, epididymis, thyroid cells, labyrinthic cells of the inner ear, and the ciliary epithelium of the eye. In addition, megalyn is expressed in the visceral yolk sac, type II pneumocytes, the parathyroid hormone (PTH)-secreting cells of the parathyroid gland, the small intestine, the endometrium, the oviduct, and the cytotrophoblast of the placenta. Megalyn has also been identified in embryonic tissues such as the trophoctodermic cells and the neuroectoderm (32, 80). During renal development, megalyn can be identified in the mesonephros, the nephronic vesicle,

and the ureteric bud (80). Megalyn is expressed in the S-shaped body later giving rise to both the glomeruli and the proximal as well as the distal tubule. Later during development, megalyn is only expressed in the proximal tubules and, to a lesser extent, the glomerulus (80).

In the kidney proximal tubule (Fig. 2), megalyn can be localized to the brush border, coated pits, endocytic vesicles (1, 3, 14, 45), and the membrane recycling compartment, dense apical tubules (6, 17, 19). The membrane expression is high in small and large endosomes in the proximal tubule cells but is virtually absent in late endosomes/prelysosomes (16). However, smaller amounts of intact and degraded megalyn have been identified in the matrix of the lysosomes (19). In rats the expression of megalyn in the proximal tubule brush border varies between different segments, with the highest expression in segment two (19). Megalyn has also been identified in the glomerular podocytes of rat kidney (44).

After translation in the rough ER (RER), megalyn binds rapidly and with high affinity to the 40-kDa protein RAP. RAP also binds other members of the LDL-receptor family and serves as a chaperone protecting newly synthesized receptor from the early binding of ligands (12, 13, 90, 94). In addition, RAP may be involved in the folding of the receptors (13). RAP deficiency is associated with a significant decrease in the expression as well as a subcellular redistribution of megalyn in the proximal tubule (10). A HNEL motif, serving as an RER-retention signal, is present in RAP, and the protein is predominantly located in this organelle (12). Thus RAP is a predominantly intracellular ligand for megalyn. However, due to its high-affinity association with megalyn, inhibiting the binding of most other ligands, it has served as an important tool for the study of ligand binding to megalyn.

Cubilin

Cubilin is highly expressed in the renal proximal tubule and the visceral yolk sac (79), the epithelium of the small intestine (9, 83), the placental cytotrophoblast (33; for a recent review, see Ref. 16), and possibly other tissues including thymus (33), although at present it seems more restricted than the expression of megalyn. In the proximal tubule (Fig. 2), cubilin expression very closely resembles that of megalyn represented by the brush border and all constituents of the coated pit endocytic and the membrane recycling pathway (78, 84). Cubilin is also identified in lysosomes (84). Similar to megalyn, cubilin is expressed in the S-shaped body during renal development whereas later expression is confined to the proximal tubule only (80). So far it has not been identified in the glomerulus.

The posttranslational processing of cubilin may involve furin-mediated cleavage in the *trans*-Golgi network, as suggested by the finding that affinity-purified human cubilin appears to be truncated at a recognition cleavage site for furin in the NH₂-terminal region (52). Furthermore, pulse-chase studies have suggested an

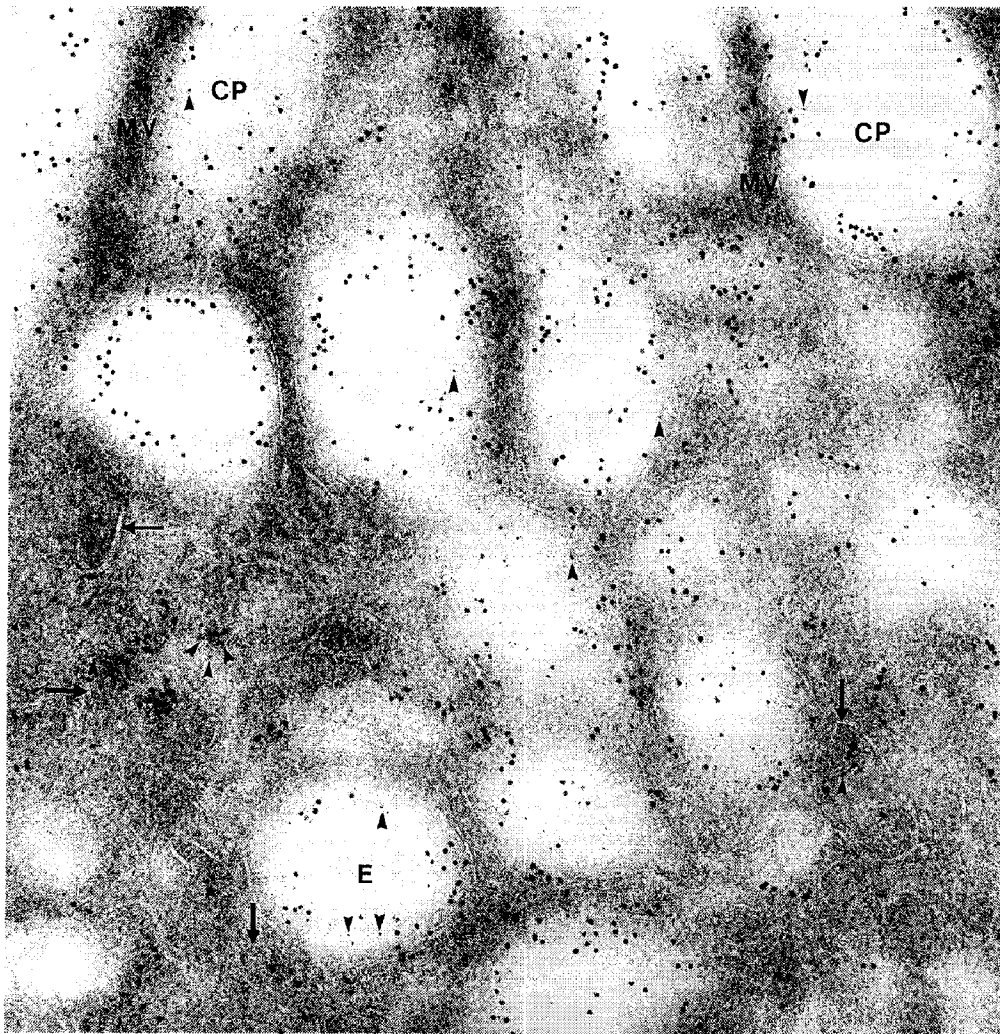


Fig. 2. Immunolocalization of megalin (10 nm gold) and cubilin (5 nm gold; arrowheads) in proximal tubule from rat. The 2 receptors are colocalized on the microvilli (MV), in coated pits (CP), apical endosomes (E), and dense apical tubules (arrows) responsible for the apical receptor recycling in these cells. Ultrathin cryosections of rat kidney cortex were incubated with polyclonal sheep anti-rat megalin and rabbit anti-rat cubilin followed by incubation with secondary antibodies coupled to colloidal gold particles. Magnification: $\times 110,000$.

unusual processing of cubilin in yolk sac cells, involving the expression of newly synthesized, endoglycosidase H-sensitive cubilin at the plasma membrane although the majority of cubilin is endoglycosidase H resistant (4). This indicates that newly synthesized cubilin is targeted to the plasma membrane and recycled to the Golgi apparatus for final processing, possibly involving both carbohydrate modifications and furin-mediated truncation. Posttranslational processing may be tissue specific, as it was recently shown that ileal cubilin undergoes more extensive NH_2 -linked glycosylation than renal cubilin (95).

Recently, it was shown that a canine disorder characterized by defective trafficking of cubilin into the apical membranes and a functional cubilin deficiency was not caused by a defect in the cubilin gene. Rather, it was suggested that a yet unknown accessory protein is required for cubilin brush-border expression (96).

FUNCTION

Protein Reabsorption

Megalyn was recognized early as a endocytic receptor involved in the tubular uptake of proteins. A large number of ligands have been identified (Table 1). Al-

though not all of these can be expected to be present in the glomerular filtrate, many are recognized markers of defective tubular reabsorption, including vitamin D-binding protein (DBP), vitamin A/retinol-binding protein (RBP), β_2 -microglobulin, and α_1 -microglobulin. Analyses in megalin-knockout mice as well as patients with Fanconi syndrome have shown several analogies in urinary protein excretion, suggesting the former to be a model of low-molecular-weight, tubular proteinuria (55). Although fewer ligands have been identified for cubilin (Table 1) so far, recent evidence strongly suggests this protein to be important for tubular protein reabsorption as well. Significant proteinuria is observed in many patients suffering from Imerslund-Gräsbech syndrome (11, 31, 40), a rare vitamin B_{12} -deficiency disease characterized by defective intestinal absorption of the vitamin B_{12} -intrinsic factor complex and recently shown in two Finnish families to be associated with mutations in the cubilin gene (2). Two mutations were identified: a point mutation causing one amino acid substitution in CUB domain 8 affecting the binding of intrinsic factor- B_{12} (2, 53); and a point mutation expected to activate a cryptic intronic splice site causing an in-frame insertion with several stop

Table 1. *Ligands to megalin and cubilin*

Megalin	Ref. No(s).	Cubilin	Ref. No(s).
<i>Vitamin-binding proteins</i>			
Transcobalamin-vitamin B ₁₂	63	Intrinsic factor-vitamin B ₁₂	9, 84
Vitamin D-binding protein	71		
Retinol-binding protein	18		
<i>Apolipoproteins</i>			
Apolipoprotein B	86	Apolipoprotein A-I	34, 51
Apolipoprotein E	91	HDL	34, 51
Apolipoprotein J/clusterin	50		
Apolipoprotein H	67		
<i>Low-molecular-weight peptides and hormones</i>			
PTH	36		
Insulin	74		
β ₂ -Microglobulin	74		
Epidermal growth factor	74		
Prolactin	74		
Lysozyme	74		
Cytochrome-c	74		
α ₁ -Microglobulin	55		
PAP-1	55		
Odorant-binding protein	55		
Transthyretin	85		
<i>Other</i>			
Albumin	23	Albumin	8
RAP	17, 42, 47, 73, 91	RAP	9
Thyroglobulin	100	Ig light chains	5
Plasminogen	41		
Lactoferrin	91		
Ca ²⁺	17		
<i>Polybasic drugs</i>			
Aminoglycosides	64		
Polymyxin B	64		
Aprotinin	64		
<i>Enzymes and enzyme inhibitors</i>			
PAI-1	87		
PAI-1-urokinase	66		
PAI-1-tPA	66, 91		
Prourokinase	87		
Lipoprotein lipase	48		
α-Amylase	10		

HDL, high-density lipoprotein; PTH, parathyroid hormone; PAP-1, pancreatitis-associated protein 1; RAP, receptor-associated protein; PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator.

codons, predicting a truncation of the receptor in CUB domain 6 (2). Thus variations in the type of mutations causing this disease may explain why some patients have selective intrinsic factor-B₁₂ malabsorption and yet no or only little proteinuria (11, 51). Intense proteinuria is likely to be caused by mutations in the

cubilin gene affecting more binding sites or result in the absence of a functional receptor. The importance of cubilin for tubular protein reabsorption is further supported by the proteinuria (27, 28), and in particular albuminuria (8), observed in dogs, characterized by a defective processing and apical insertion of cubilin within the epithelial cells (Fig. 3), causing a vitamin B₁₂ malabsorption syndrome (27, 28).

Thus structural as well as functional defects in either megalin or cubilin are associated with proteinuria, suggesting both receptors to be essential to normal tubular reabsorption of filtered proteins.

Ligands. The known ligands for megalin normally filtered in the glomeruli include DBP, RBP, the vitamin B₁₂/cobalamin plasma carrier protein, transcobalamin (TC)-B₁₂, PTH, insulin, EGF, prolactin, albumin, β₂- and α₁-microglobulin, apo H, transthyretin, lysozyme, cytochrome-c, α-amylase, and Ca²⁺ (Table 1). Many of these proteins are either carrier proteins or hormones, suggesting megalin to be involved in the metabolism of vitamins, and in the renal clearance of many filtered hormones by endocytic uptake and degradation. In most cases ligand binding is Ca²⁺ dependent, and megalin itself binds calcium very strongly (17). It has been shown by site-directed mutagenesis analysis that mutations of basic amino acid residues in aprotinin, a 6-kDa proteinase inhibitor and a ligand for megalin, decrease the affinity for the receptor, suggesting that binding is charge dependent and favored by cationic sites on the ligands (64). However, many ligands are anionic proteins, indicating that it is the distribution of charge rather than the overall isoelectric point that is important for binding, as also suggested previously (20). The binding of almost all ligands can be inhibited by RAP. This high-affinity binding is exploited in both the study of receptor function, ligand binding, as well as for affinity purification of the receptor (66). Megalin has at least two different binding sites for RAP, as demonstrated by surface plasmon resonance analysis. Binding of the first RAP molecule with high affinity was followed by binding of a second molecule with lower affinity (63).

On the basis of immunoprecipitation studies, it was suggested that megalin complexes with the sodium-hydrogen exchanger, NHE-3, in the kidney proximal tubule brush border and that megalin could be involved in the regulation of this transporter by mediating endocytosis (7). Still, further evidence is needed to confirm this hypothesis.

The known ligands for cubilin expected to be present in normal glomerular ultrafiltrate so far only include albumin, immunoglobulin light chains, and apo A-I. Albuminuria is an important marker for renal disease, and a recent study has shown that cubilin is essential to normal renal tubular reabsorption of filtered albumin (8). This, as well as the binding of myeloma light chains (5), may implicate a role for cubilin in the progression of renal disease. Several studies have suggested that tubular uptake of an increased load of filtered proteins, including albumin, contributes to the development of tubular and interstitial inflammation

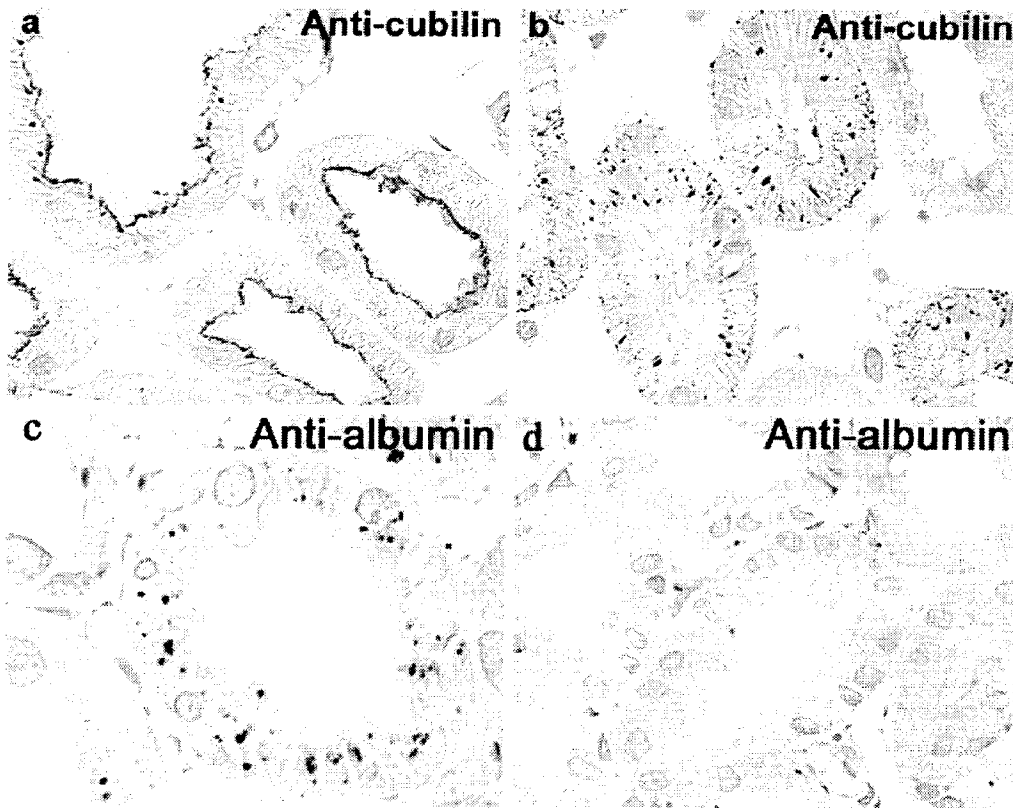


Fig. 3. Immunolocalization of cubilin and endogenous albumin in proximal tubules from the kidney of normal dogs (a and c) and dogs with a defective processing and insertion of cubilin into the apical plasma membrane (b and d). In contrast to normal dogs (a), no cubilin can be identified at the luminal plasma membrane of the affected dogs (b), resulting in a functional cubilin deficiency. As a result, no labeling for albumin is observed in the tubules of the affected dogs (d), reflecting the defective tubular reabsorption of filtered albumin. In normal dogs (c), reabsorbed albumin can be identified in vesicular structures within the proximal tubule cells. Semithin cryosections of dog kidney cortex were incubated with either polyclonal anti-dog cubilin or polyclonal anti-human albumin, followed by peroxidase-labeled secondary antibody and visualization by incubation with diaminobenzidine and H_2O_2 . Magnification: $\times 500$ (a, b, and d); $\times 1,000$ (c).

and fibrosis. Thus cubilin-mediated tubular uptake of albumin during states of hyperfiltration of proteins may be an important factor for the development and progression of chronic renal disease. Cubilin also binds RAP (9) although the significance of this is not determined. The binding of apo A-I suggests a role in the renal degradation of this apolipoprotein (34, 51). The kidney is the major site for the catabolism of apo A-I, involving glomerular filtration and subsequent tubular uptake.

Most if not all of the ligands taken up by megalin or cubilin in the proximal tubule are degraded in lysosomes. So far, no evidence for the transcellular transport of protein ligands, including carrier proteins, has been published. However, vitamins, steroids, fatty acids, and other substances carried by proteins, filtered and reabsorbed by the receptors, and released within the cell most likely are transported back to the circulation, possibly after biochemical modifications, as discussed later. Megalin-mediated transcellular transport of proteins has been demonstrated in other tissues such as the thyroid gland for thyroglobulin (60, 61) and in cerebral vascular endothelium and in the choroid epithelium for apo J (101).

Megalin-knockout mice. Much of our recent knowledge on the functions of megalin is based partially on data recovered from the study of megalin-deficient mice. These mice, produced by gene targeting, exhibit severe forebrain abnormalities as well as lung defects (92). Most of them die perinatally; however, approximately 1 of 50 survive to adulthood, constituting a model for the study of megalin function (92). The kid-

neys of these mice are generally normal; however, ultrastructurally the proximal tubule cells are characterized by a loss of apical endosomes (92), coated pits, and recycling dense apical tubules (21). This probably reflects decreased endocytic activity and supports an important general role for megalin in maintaining proximal tubule endocytosis. The megalin-deficient mice excrete an increased amount of a number of low-molecular-weight plasma proteins in the urine. This is a result of defective tubular reabsorption, as shown by the absence of immunodetectable protein ligands in the proximal tubule cells of deficient mice (8, 18, 36, 55, 67, 71). So far, no significant changes in transport of water, electrolytes, glucose, or amino acids have been described in megalin-deficient mice (55).

Receptor-receptor interactions. As discussed previously, the primary sequence of cubilin does not predict a transmembrane domain (65). Thus cubilin itself does not harbor any obvious sites for interaction with adaptor proteins or other mediators of clathrin-coated endocytosis. However, a high-affinity, Ca^{2+} -dependent, and partially (75%) RAP-inhibitable binding between purified cubilin and megalin has been described (65), suggesting that megalin mediates the cointernalization and possibly recycling of cubilin. The binding between megalin and cubilin appears to be complex. However, by fitting the binding data to a one-binding-site model, a dissociation constant of ~ 7 nM was measured (65). A similar mechanism involving another member of the LDL-receptor family has been suggested for the internalization of the urokinase receptor-bound-urokinase-inhibitor complex (22). This glyco-

phosphatidylinositol-anchored receptor-ligand complex is internalized by binding to LRP. Recently, it was shown in vitro that the uptake of high-density lipoprotein, which binds to cubilin, was inhibited by anti-megalin antibodies as well as by megalin anti-sense oligonucleotides (33). In addition, treatment with megalin anti-sense oligonucleotides also reduced the surface expression, but not total expression, of cubilin (33). This may indicate that megalin is also involved in trafficking of cubilin.

In addition to direct receptor interaction, megalin and cubilin seem to share ligands. So far, these include RAP and albumin (Table 1). Thus in the case of albumin both megalin and cubilin are involved in the endocytic uptake (Fig. 1) (8, 98). This may include direct binding of albumin to both receptors as well as receptor-receptor interaction after binding to cubilin.

Vitamin Metabolism and Homeostasis

The megalin-mediated tubular reabsorption of vitamin-carrier proteins appears important for both maintaining vitamin homeostasis and metabolizing certain vitamins, notably the renal hydroxylation of vitamin D. So far, three vitamin-carrier proteins (Table 1), all of which are filtered in the glomeruli, have been identified as ligands for megalin. These are DBP, RBP, and TC-B₁₂. DBP (71) and RBP (18) were both initially identified in the urine of megalin-deficient mice, and TC, which previously was identified as a ligand for megalin (63), has also subsequently been demonstrated in the urine of these mice (Birn H, Willnow T, Nielsen R, Norden AGW, Moestrup S, Nexø E, and Christensen E, unpublished observations).

Filtered plasma vitamin D carrier protein DBP binds to megalin, which mediates the endocytosis of this protein. The megalin-mediated uptake of 25-(OH) vitamin D₃ in the proximal tubule is followed by lysosomal degradation of DBP and subsequent conversion of 25-(OH) vitamin D₃ to 1,25-(OH)₂ vitamin D₃, which is then returned to the circulation (71). Considerable amounts of DBP and 25-(OH) vitamin D₃ are excreted in the urine of megalin-deficient mice (71). In addition, these mice have reduced plasma vitamin concentration. Furthermore, especially the young mice suffer from severe bone calcification abnormalities, indicating that megalin-mediated tubular uptake is essential for normal calcium homeostasis in these animals (71).

Megalin also mediates the reabsorption of RBP (18) and TC (63) by endocytosis. This must be followed by release of internalized vitamins to the circulation to maintain vitamin homeostasis. Increased amounts of both TC and vitamin B₁₂ can be identified in the urine of megalin-deficient mice in combination with reduced kidney concentrations of B₁₂ (Birn H, Willnow T, Nielsen R, Norden AGW, Moestrup S, Nexø E, and Christensen E, unpublished observations). This shows that megalin is important for preventing urinary loss of the vitamins. In addition, the kidney may serve a vitamin B₁₂ storage function, possibly involving megalin-mediated uptake (Birn H, Willnow T, Nielsen R,

Norden AGW, Moestrup S, Nexø E, and Christensen E, unpublished observations). It has been estimated that the tubular vitamin B₁₂ reabsorption is similar to the intestinal uptake (56).

Although the basic molecular mechanisms for the efficient tubular clearance of the carrier proteins appear well established, the subsequent intracellular handling of the vitamins remains to be clarified. After release of vitamins, the carrier proteins most likely are degraded in lysosomes. The vitamins may be transported into the cytoplasm by either diffusion through the vesicular membranes or facilitation by a transport protein. A membrane-associated vitamin B₁₂ transporter has been described (39, 77). Vitamin D₃ subsequently undergoes hydroxylation to a more active form, and, similarly, cobalamin may be metabolized into other forms. The final steps, i.e., the release of vitamin and possible coupling to plasma carrier proteins, have not been elucidated. Because vitamin D₃ is lipophilic, it has been hypothesized that the vitamin diffuses through the basolateral membranes and meets its carrier protein DBP extracellularly (71). Alternatively, it has been suggested that reabsorbed retinol or B₁₂ is coupled to newly synthesized RBP or TC within the proximal tubule cells and then secreted as a complex (18, 70). These pathways are summarized in Fig. 4.

The importance of megalin for the uptake and transport of certain vitamins may be indicated by the fact that some of the defects seen in the megalin-deficient mice resemble syndromes of vitamin deficiency. Megalin is located in the visceral epithelium of the yolk sac (15, 25), important for early embryonic nutrition in rodents, and in addition is also in the cytotrophoblast of the placenta (58). Thus megalin may be involved in the transport of vitamins from the maternal circulation to the embryo. It has been hypothesized that megalin and possibly other members of the LDL-receptor family serve an important function mediating tissue-specific uptake of carrier-bound retinoids and steroids, including vitamins and hormones (93). This would provide a mechanism by which certain cell types could accumulate large quantities of steroids beyond what is possible solely by diffusion of free hormones.

Calcium

Megalin serves important functions in calcium homeostasis. As described, megalin mediates proximal tubule endocytosis of the DBP-vitamin D complex, resulting in the renal hydroxylation and activation of vitamin D (71). In addition, megalin mediates tubular uptake and subsequent lysosomal degradation of PTH (36). It has been suggested that this may regulate the amount of PTH available for stimulation of the tubular PTH/PTH-related peptide receptor (36). Megalin is also expressed on the PTH-secreting cells in the parathyroid gland (57) and is, in itself, a very strong calcium binder (17). Thus it may be speculated that the protein serves as a calcium sensor in both the parathyroid gland and the kidney.

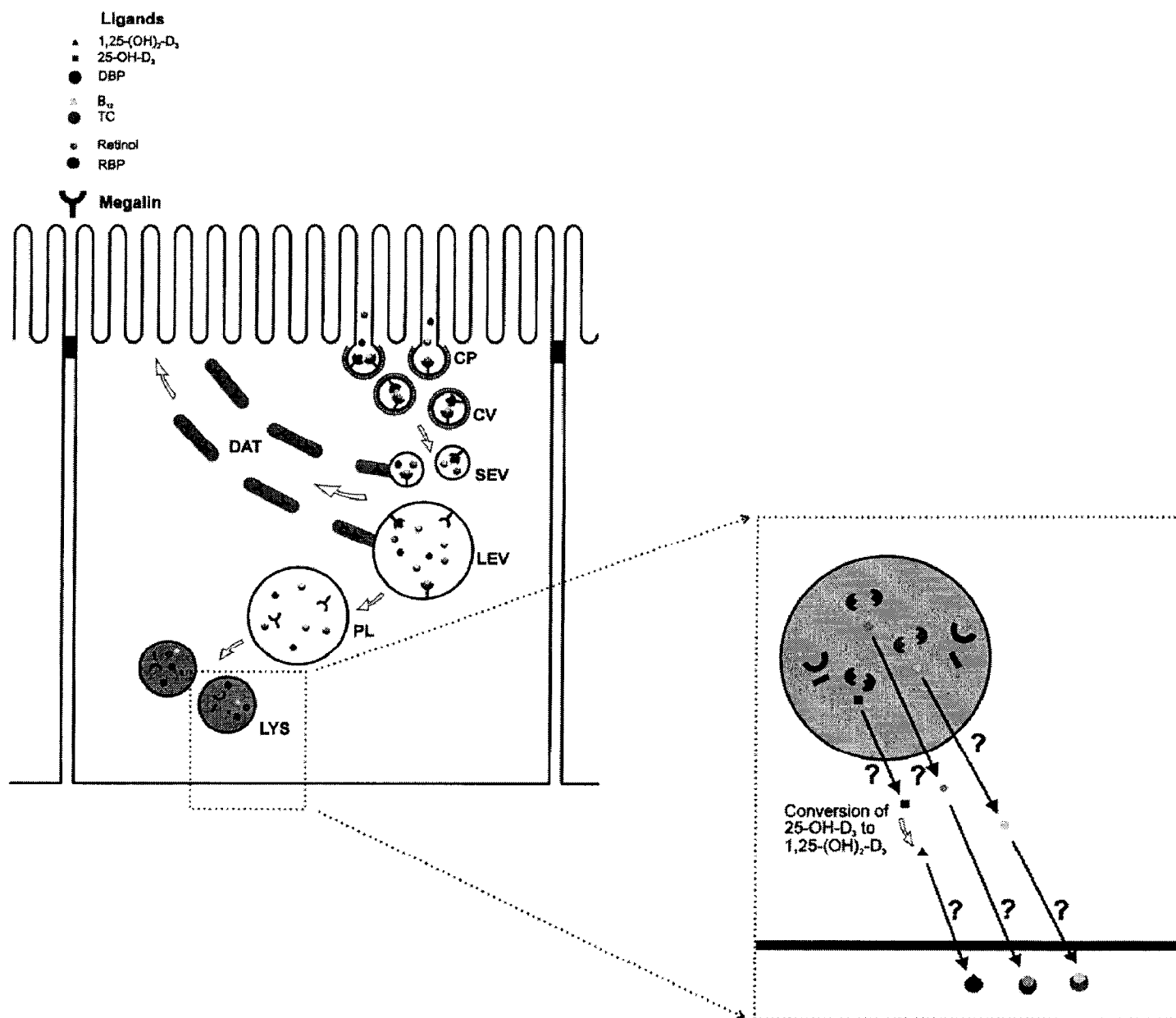


Fig. 4. Schematic illustration of the megalin-mediated uptake of the 3 vitamin carrier protein complexes: DBP-vitamin D₃, TC-vitamin B₁₂, and RBP-vitamin A in renal proximal tubule. After megalin-mediated endocytosis via apical coated pits (CP), the complexes accumulate in lysosomes (LYS) for degradation of the proteins, while the receptor is returned to the apical plasma membrane via dense apical tubules (DAT). The intracellular processing of the vitamins may include modifications such as hydroxylation of 25-OH-D₃ to 1,25-(OH)₂-D₃ before basolateral secretion. The mechanisms for secretion of the 3 vitamins remain to be clarified. CV, coated vesicles; SEV, small endocytic vesicles; LEV, large endocytic vesicles; PL, prelysosomes.

Drugs

Megalin has been shown to bind and mediate proximal tubule uptake of several polybasic and potential nephrotoxic substances, including the aminoglycosides gentamicin, netilmicin, and amikacin, as well as polymyxin B (64). These drugs are readily filtered in the glomeruli followed by endocytic uptake and accumulation in the endocytic apparatus and lysosomes of the proximal tubule cells (68, 69, 89). Binding to phospholipids in the apical brush-border membrane has been implicated in the tubular uptake (82); however, the

affinity of these drugs for megalin is higher than for phosphatidylinositol, suggesting that aminoglycosides bound to membrane lipids are transferred to megalin for endocytosis (64). Also, the expression of megalin in the labyrinthic cells of the inner ear is intriguing (62, 97) because aminoglycosides as well as polymyxin B are also known to be ototoxic.

Heymann Nephritis

Megalin was originally identified as the antigen in Heymann nephritis (43), a rat model of human mem-

branous glomerulonephritis (reviewed in Ref. 26). Circulating anti-megalin antibodies bind to megalin expressed in glomerular podocytes, causing destruction of the basement membrane.

So far, no anti-megalin antibodies have been associated with any human renal disease. However, circulating anti-megalin antibodies have been identified in serum from patients with autoimmune thyroiditis as well as some other thyroid diseases (59). Whether these antibodies are involved in the pathogenesis of the underlying autoimmune disease remains to be established.

Signaling Functions?

The megalin cytoplasmic domain contains several regions, suggesting a possible signaling function in addition to its role as endocytic receptor. These include several Src homology 3 and one Src homology 2 recognition sites as well as the NPXY motifs. So far, no definite evidence for the involvement of megalin in signal transduction has been published. However, other members of the LDL-receptor family, the VLDL receptor and apo E receptor-2, have been suggested to be involved in signal transmission initiated by the extracellular matrix protein reelin in the cerebral cortex and cerebellum (88; for recent reviews, see Refs. 35 and 93). This signaling requires the intracellular mammalian disabled protein 1 (Dab1), which was shown to bind to the NPXY motifs on the cytoplasmic tail of both receptors. After binding of Dab1 to the cytoplasmic tail of the VLDL-receptor or apo E receptor-2, Dab1 may be phosphorylated on tyrosine residues, allowing binding and activating nontyrosine kinases. Dab1 has been shown to bind to other members of the LDL-receptor family (38). Also, by using a yeast two-hybrid system, it was recently shown that the cytoplasmic tail of megalin binds the cytosolic disabled protein 2 (Dab2) (72) as well as a number of other cytoplasmic proteins with a potential signaling function (30). Although no specific cellular response has been associated with these interactions involving megalin, Dab2 was identified in rat kidney by Western blotting and was coprecipitated with megalin by using both anti-Dab2 and anti-megalin antibodies (72). This indicates a potential signaling pathway involving megalin in the kidney.

Thus several lines of evidence indicate that members of the LDL-receptor family may be involved in signal transduction although no specific response or overall pathway has been identified for megalin.

CONCLUSIONS

Megalin and cubilin constitute two large, endocytic receptors heavily expressed in the endocytic apparatus of the kidney proximal tubule. Absence or dysfunction of either receptor is associated with significant tubular proteinuria, showing that both are important for normal absorption of filtered proteins, including albumin. Although structurally very different, both receptors may be functionally linked. Some ligands are common to both receptors, and megalin-cubilin interaction

seems to be important for the endocytosis and recycling of the "peripherally attached" cubilin. Megalin is important for tubular uptake and metabolism of several hormones and vitamin-carrier protein complexes, including the renal activation by hydroxylation of vitamin D. In addition, megalin is involved in the tubular uptake of potential nephrotoxic drugs, including aminoglycosides. Thus modification of receptor function may be a valuable prospect of future research. This is encouraged by findings suggesting that tubular uptake of an increased load of filtered proteins, including albumin, may contribute to the progression of chronic renal disease. Finally, there is new evidence suggesting that megalin may be involved in signal transduction. No doubt, future studies will help to unfold this potential new aspect of megalin receptor function.

This work was supported by grants from the Danish Medical Research Council, the Novo Nordic Foundation, the Danish Biotechnology Program, and University of Aarhus Research Foundation.

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An Endocytic Pathway Essential for Renal Uptake and Activation of the Steroid 25-(OH) Vitamin D₃

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Summary

Steroid hormones may enter cells by diffusion through the plasma membrane. However, we demonstrate here that some steroid hormones are taken up by receptor-mediated endocytosis of steroid-carrier complexes. We show that 25-(OH) vitamin D₃ in complex with its plasma carrier, the vitamin D-binding protein, is filtered through the glomerulus and reabsorbed in the proximal tubules by the endocytic receptor megalin. Endocytosis is required to preserve 25-(OH) vitamin D₃ and to deliver to the cells the precursor for generation of 1,25-(OH)₂ vitamin D₃, a regulator of the calcium metabolism. Megalin^{-/-} mice are unable to retrieve the steroid from the glomerular filtrate and develop vitamin D deficiency and bone disease.

Introduction

Vitamin D₃ is a steroid hormone that plays an important role in regulation of the systemic calcium and bone metabolism. It is produced in the skin from the precursor 7-dehydrocholesterol and released into the circulation. Subsequently, vitamin D₃ undergoes two sequential hydroxylation reactions: first in the liver to 25-(OH) vitamin D₃ and then in the kidney to 1,25-(OH)₂ vitamin D₃. The latter is the active form of the vitamin that acts through binding to nuclear vitamin D receptors expressed in various target tissues. In particular, 1,25-(OH)₂ vitamin D₃ induces intestinal absorption of calcium from the diet to increase systemic Ca²⁺ concentrations.

Like other steroid hormones, vitamin D₃ metabolites are lipophilic compounds transported in the circulation by plasma carrier proteins. The 58 kDa vitamin D-binding protein (DBP) is the principal transporter for vitamin D molecules and exhibits highest affinity for 25-(OH)

vitamin D₃ (K_d of 10⁻¹⁰–10⁻¹² M) (Haddad et al., 1981; Haddad, 1995). Due to this tight binding and the high plasma concentration of DBP (0.3–0.5 mg/ml), virtually all 25-(OH) vitamin D₃ molecules in the circulation are present in a complex with DBP. Only approximately 0.003% of the metabolite is found in free form (Bikle et al., 1986).

The regulated step in generation of active vitamin D is the conversion of 25-(OH) vitamin D₃ to 1,25-(OH)₂ vitamin D₃ in the kidney. The cell type responsible for this activation is the epithelial cell of the proximal tubule. This cell type takes up the precursor 25-(OH) vitamin D₃ and converts it into the active vitamin by action of the 25-(OH) vitamin D₃ 1 α -hydroxylase in the mitochondria (Takeyama et al., 1997). Considerable interest has focused on elucidating the specific mechanisms that deliver 25-(OH) vitamin D₃ to this specialized cell type. In particular, the mode of uptake of the steroid and the role of DBP in this process is still unclear. According to the "free hormone hypothesis," it is the free 25-(OH) vitamin D₃ that is taken up into the tubular epithelium. Uptake is believed to proceed from the basolateral site of the tubule by yet unknown mechanisms (Mendel, 1989; Haddad, 1995).

Recently, a major pathway for the uptake of ligands from the luminal site into the proximal tubules was identified. Megalin is a multifunctional clearance receptor expressed in the neuroepithelium and in proximal tubular cells of the kidney. The protein shares extensive structural similarities with the low density lipoprotein (LDL) receptor and is a member of the LDL receptor superfamily (Saito et al., 1994). Like other members of this gene family, megalin exhibits broad ligand specificity and mediates the uptake and lysosomal degradation of numerous macromolecules. Ligands for megalin identified *in vitro* include lipoproteins, proteases, and protease inhibitors (Willnow et al., 1992; Moestrup et al., 1995, 1998; Stefansson et al., 1995). The expression of megalin on the luminal surface of the tubular epithelium and its ability to take up ligands injected into the proximal tubules suggests a role for the receptor in the clearance of filtered macromolecules (Moestrup et al., 1995, 1996). The nature of endogenous ligands taken up by megalin *in vivo* remains unclear.

To elucidate the physiological significance of megalin, we recently generated mice deficient in the encoding gene. Most megalin-deficient mice die perinatally from holoprosencephaly, a developmental defect of the forebrain (Willnow et al., 1996). The exact causes underlying this defect have not been elucidated, but the severity of the phenotype varies among individual animals, and 1 in 50 of the megalin^{-/-} mice survive to adulthood. Surviving knockout animals were used to study the role of the receptor in the renal proximal tubules. Unexpectedly, we found that complexes of 25-(OH) vitamin D₃ and DBP are filtered through the glomerulus and reabsorbed by megalin into the proximal tubular cells. Abnormal urinary excretion of 25-(OH) vitamin D₃ and DBP in megalin knockout mice results in severe vitamin D deficiency and bone disease. Thus, we have identified

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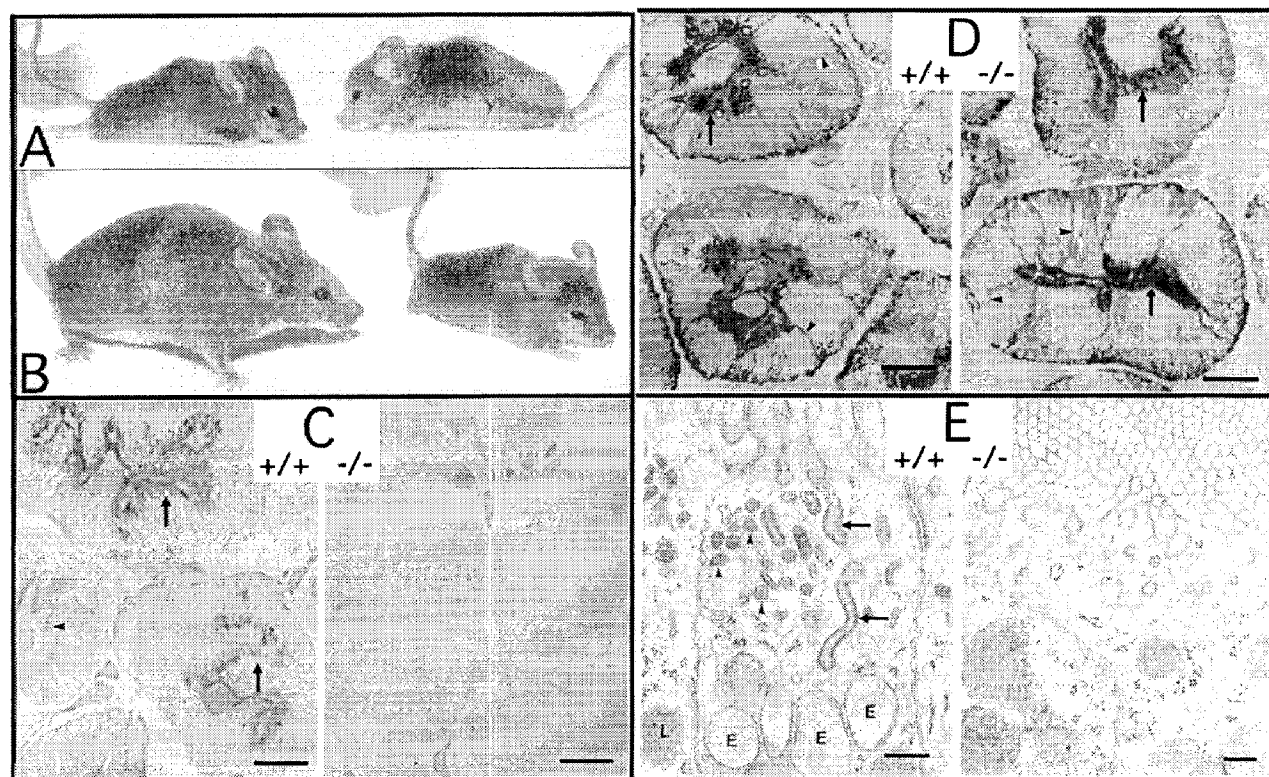


Figure 1. Appearance and Kidney Histology of Wild-Type and Megalin^{-/-} Mice

(A) Two megalin^{-/-} mice are shown at 6 weeks of age. Notice facial malformations indicating holoprosencephaly.

(B) Depicted are a megalin^{-/-} mouse (right) and wild-type littermate.

(C and D) The immunohistochemical detection of megalin (C) and aquaporins (D) in wild-type (+/+) and megalin^{-/-} kidneys (-/-) is shown. Semithin cryosections of tissues fixed in 4% paraformaldehyde were incubated with primary antibodies followed by peroxidase-conjugated secondary antibody. Expression of megalin and aquaporins on the apical surface of the epithelium is indicated by arrows; arrowheads denote basolateral expression of aquaporins. Bars = 10 μ m.

(E) Electron micrographs of sections through proximal tubules of wild-type and megalin-deficient kidneys are shown. Tissues were fixed in 4% paraformaldehyde and 1% glutaraldehyde, postfixed in OsO₄, dehydrated, and embedded in Epon prior to routine sectioning. Arrowheads indicate dense apical tubules; arrows indicate coated pits. E, endosomes; L, lysosomes. Bars = 0.25 μ m.

a renal uptake pathway that is essential to preserve vitamin D metabolites and to deliver the precursor for generation of 1,25-(OH)₂ vitamin D₃.

Results

Urinary Excretion of Vitamin D-Binding Protein in Megalin^{-/-} Mice

Approximately 2% of megalin knockout animals survive to adulthood. These animals are severely retarded in growth (Figures 1A and 1B). Superficially, kidneys from the adult megalin^{-/-} mice were indistinguishable from those of wild-type controls. In particular, the lack of receptor expression in the proximal tubular epithelium (Figure 1C) did not affect expression of other cell surface proteins such as aquaporins (Figure 1D), insulin-like growth factor-II receptor, or ecto-5'-nucleotidase (not shown). However, electron microscopical analysis demonstrated that components of the endocytic apparatus, including endosomes, lysosomes, and recycling vesicles (dense apical tubules) were significantly reduced in number (Willnow et al., 1996). This result suggested a deficiency in the uptake of filtered macromolecules in proximal tubules lacking megalin (Figure 1E). To test this

hypothesis, we analyzed the urine of megalin-deficient mice by SDS polyacrylamide gel electrophoresis (PAGE) and compared it to control samples (Figure 2). No proteins larger than serum albumin (68 kDa) were present in the urine, demonstrating normal glomerular function in megalin^{-/-} animals. However, knockout mice specifically excreted several low-molecular-weight proteins that were not found in control samples (Figure 2A). Such low-molecular-weight proteinuria indicates an inability of the proximal tubules to reabsorb small plasma proteins filtered through the glomerulus. To identify these proteins, which are potential ligands for megalin, an amino-terminal peptide sequence was derived from the major 58 kDa protein found in knockout urine (Figure 2A). The sequence obtained was identical to the first eight amino acids (L E R G R D Y E) of the mature mouse DBP (Yang et al., 1990). DBP was present exclusively in urine from megalin-deficient but not control animals (Figure 2B).

Megalin Is the Renal DBP Receptor

To test directly whether megalin binds DBP, the carrier was purified from human serum and from urine of patients with Fanconi syndrome, who are known to excrete

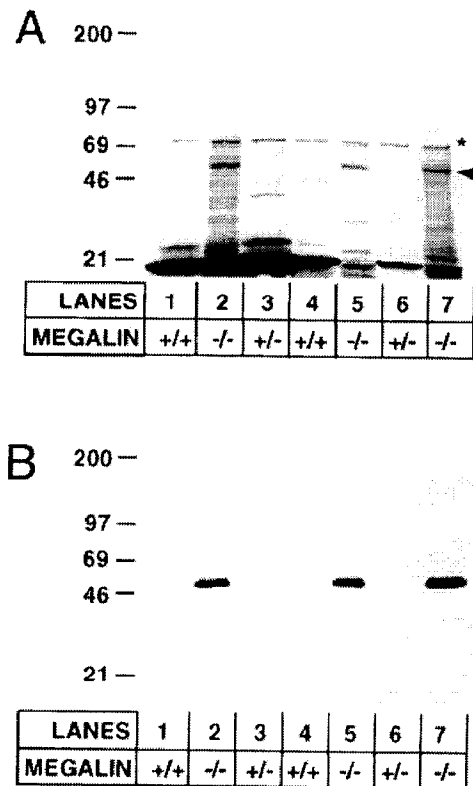


Figure 2. Urinary Protein Profile of Wild-Type and Megalin^{-/-} Mice (A) Fifteen microliters of urine from mice of the indicated genotypes were subjected to 4%–15% nonreducing SDS-PAGE and staining with Coomassie. Protein bands corresponding to serum albumin (asterisk) and DBP (arrowhead) are highlighted. (B) Urine samples as in (A) were subjected to immunoblot analysis using anti-DBP antiserum and the enhanced chemiluminescence system (ECL).

DBP (Figure 3A, inset). In BIAcore experiments, urine and serum DBP bound specifically to megalin with a K_d of $108 \text{ nM} \pm 62 \text{ nM}$ (standard error of the mean, $n = 4$) (Figure 3A). Binding was dependent on calcium, a characteristic feature of ligand binding to LDL receptor-related receptors. Complexes of DBP and 25-(OH) vitamin D₃ formed in vitro bound to the receptor with similar affinity as DBP alone (K_d of $111 \mu\text{M} \pm 44 \mu\text{M}$, $n = 4$, data not shown). To show that megalin is the only cellular binding site for DBP in the kidney, cryosections of wild-type mouse kidneys were incubated with purified DBP. Binding of the protein colocalized with megalin on the apical surface of the proximal tubular cells. No binding was seen in megalin-deficient kidneys (Figure 3B). When anti-DBP antiserum was added to wild-type kidney sections, endogenous DBP was detected in endosomes and lysosomes, indicating uptake from the glomerular filtrate. In contrast, no uptake of endogenous DBP was detected in knockout kidneys (Figure 3C). The complete absence of DBP binding sites and lack of uptake of endogenous DBP in knockout kidneys indicated that megalin is the only receptor to retrieve the protein from the glomerular filtrate.

Megalin Mediates Tubular Uptake of DBP and 25-(OH) Vitamin D₃/DBP Complexes

To show that megalin mediates endocytosis of DBP from the glomerular filtrate, ¹²⁵I-DBP was infused into

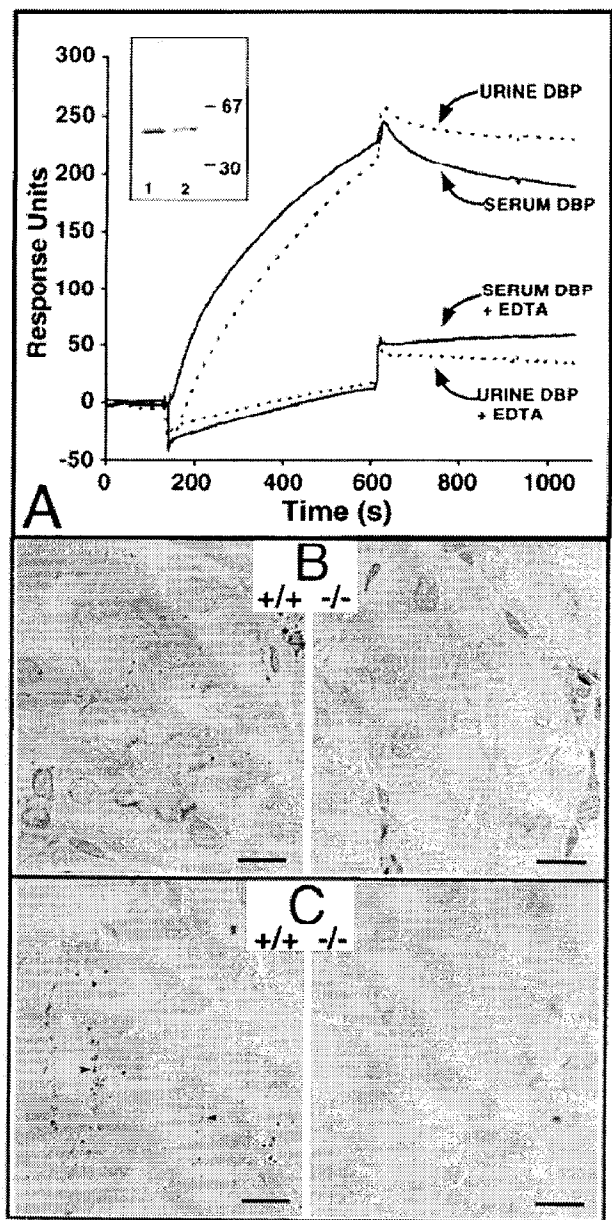


Figure 3. Analysis of DBP Binding to Megalin

(A) DBP purified from human serum or urine of patients with Fanconi syndrome was incubated with immobilized megalin in the presence or absence of 20 mM EDTA. Binding to the receptor was detected by surface plasmon resonance signal (BIAcore) as described in Experimental Procedures and is indicated in response units. (Inset) Preparations of DBP from human serum (lane 1) and urine (lane 2) are depicted. DBP was purified by immunoaffinity chromatography using anti-DBP antiserum coupled to CNBr-activated sepharose. One microgram of DBP was subjected to 10% reducing SDS-PAGE and staining with AgNO₃.

(B) Cryosections through wild-type and megalin^{-/-} kidneys were incubated with purified serum DBP, and cell surface binding of DBP was detected using rabbit anti-DBP antiserum and peroxidase-conjugated anti-rabbit IgG. Bars = 10 μm .

(C) Cryosections through wild-type and megalin^{-/-} kidneys were incubated with anti-DBP IgG, followed by peroxidase-conjugated anti-rabbit IgG. Arrowheads indicate endogenous DBP present in endosomes and lysosomes of wild-type kidneys. Bars = 10 μm .

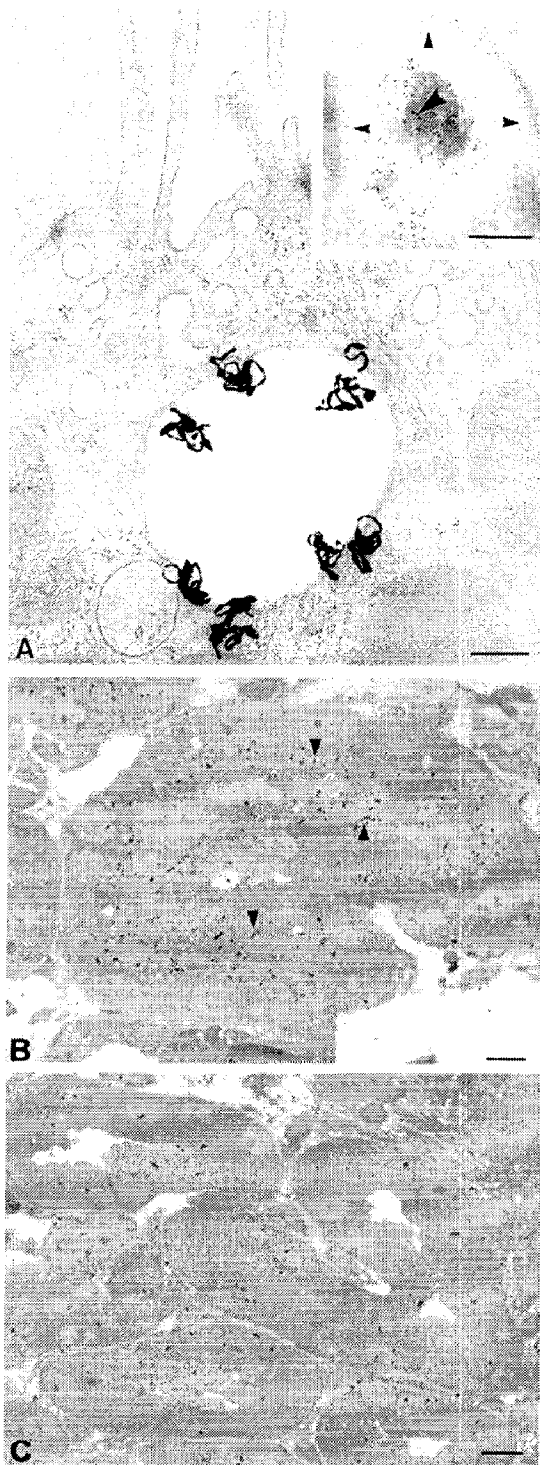


Figure 4. Inhibition of Proximal Tubular Uptake of ^{125}I -DBP by the Receptor-Associated Protein

(A) Individual rat proximal tubules were microinfused in vivo with 50 nl of phosphate buffer containing 0.5–2.7 pmol ^{125}I -DBP (1000 cpm total) in the absence or presence of 0.4 mg/ml RAP. The amount of tubular uptake of ^{125}I -DBP was determined as depicted in Table 1. Then the kidneys were prepared for routine sectioning as in Figure 1E and the electron micrographs subjected to autoradiography. In the absence of RAP, ^{125}I -DBP was detected in apical endosomes of proximal tubular cells (shown). No cellular uptake of ^{125}I -DBP was observed in the presence of RAP (not shown). Bar = 0.25 μm . (Inset) The inset illustrates lysosomal accumulation of DBP by incubation of ultrathin sections with rabbit anti-DBP and mouse anti-LAMP1

Table 1. Uptake of ^{125}I -DBP into Perfused Rat Proximal Tubules

Ligand	Percent Renal Uptake (\pm SEM)	n
^{125}I -DBP	75.0 ± 11.3	8
^{125}I -DBP + RAP	8.2 ± 10.4	8

Percent of total radioactivity taken up into proximal tubules is indicated. For details, see Experimental Procedures. n, number of injected tubules; SEM, standard error of the mean.

proximal convoluted tubules of anesthetized rats and the amount of tubular uptake of the tracer was determined. In these experiments, a total of 75% of the injected ligand was taken up (Table 1). The internalized DBP was detected in intracellular vesicles that also contained LAMP1, a lysosomal marker protein, suggesting that the protein was delivered to lysosomal degradation (Figure 4A). To show that megalin was responsible for the endocytosis of DBP, we applied a well-established antagonist to inhibit megalin activity in the tubules. The receptor-associated protein (RAP) is a 39 kDa protein that specifically prevents binding of ligands to LDL receptor-related receptors and that has been used previously to block megalin activity in vivo. In these studies, RAP did not affect other known endocytic receptors in proximal tubular cells (Moestrup et al., 1995, 1996). By incubation of wild-type and knockout kidney sections with radiolabeled RAP, megalin was found to be the only RAP-binding protein in the kidney (Figures 4B and 4C). When ^{125}I -labeled DBP was microinfused into proximal tubules in the presence of RAP, only 8.2% of the ligand was taken up (Table 1), whereas the remainder was excreted into the urine. Receptor-mediated uptake and lysosomal degradation of ^{125}I -DBP was also confirmed in cultured BN16 choriocarcinoma cells expressing megalin. In these cells, $18\% \pm 0.01\%$ of the added ^{125}I -DBP was degraded within 4 hr. Degradation was inhibited significantly by RAP ($3.9\% \pm 0.01\%$) or chloroquine ($8.3\% \pm 0.01\%$). Combined application of both inhibitors had no additive effect ($2.4\% \pm 0.1\%$ ligand degraded in 4 hr).

Given the ability of megalin to clear DBP from the glomerular filtrate, we next tested whether DBP and 25-(OH) vitamin D_3 /DBP complexes are continuously filtered through the glomerulus and reabsorbed by this receptor in the proximal tubules. ^{125}I -labeled DBP was infused directly into the renal arteries of rats, and the uptake of radioactivity in kidney, blood, and urine was determined (see Experimental Procedures). Sixty minutes after infusion, 86.5% of the radioactivity was found in the circulation as proteolytic degradation products (Table 2). In contrast, when megalin activity was blocked

antibodies followed by gold anti-rabbit and anti-mouse IgG. DBP, large gold particles, large arrowheads; LAMP1, small gold particles, small arrowheads.

(B and C) Semithin cryosections through wild-type (B) and megalin knockout kidneys (C) were incubated with ^{125}I -labeled RAP and subjected to autoradiography. The arrowheads denote ^{125}I -RAP bound to the luminal surface of proximal tubular cells in wild-type kidneys (B). No RAP binding was seen in megalin-deficient kidneys (C). Bars = 5 μm .

Table 2. Uptake of ¹²⁵I-DBP and ³H-25-(OH) Vitamin D₃/DBP into Perfused Rat Kidneys

Ligand	t (Min)	n	Percent Recovered Ligand (± SEM)		
			Urine	Kidney	Blood
¹²⁵ I-DBP	60	5	7.6 ± 3.3	5.8 ± 2.4	86.5 ± 5.9
¹²⁵ I-DBP + RAP	60	4	84.2 ± 7.6	2.4 ± 2.0	13.4 ± 7.8
³ H-25-(OH) D ₃ /DBP	60	5	7.4 ± 6.7	7.8 ± 4.6	84.6 ± 6.2
³ H-25-(OH) D ₃ /DBP + RAP	10	1	6.8	0.6	92.6
	30	2	41.7 ± 11	3.8 ± 0.6	54.5 ± 10.2
	60	4	86.1 ± 11	0.05 ± 0.02	13.9 ± 11.2

Percent of total radioactivity recovered in blood, kidney, and urine at the indicated time points after injection of the ligand is shown. For details, see Experimental Procedures.

n, number of animals; SEM, standard error of the mean; t, perfusion time in minutes.

by coinjection of RAP, only 13.4% of ¹²⁵I-DBP was detected in the plasma, while 84.2% was excreted into the urine. A similar experiment was performed using complexes of ³H-25-(OH) vitamin D₃ and unlabeled DBP. To follow the kinetics of glomerular filtration, the ligand was allowed one pass (10 min perfusion) or several passes (30 or 60 min perfusion) through the kidney (see Experimental Procedures). Like ¹²⁵I-DBP, ³H-25-(OH) vitamin D₃ also appeared in the urine when megalin function was blocked by coinfusion of RAP (Table 2). The amount of the vitamin excreted correlated directly with the time of perfusion. Thus, 6.8% of the tracer was filtered per passage through the kidney. After continued perfusion for 60 min, virtually all ³H-25-(OH) vitamin D₃ was recovered in the urine (86.1%). When ³H-25-(OH) vitamin D₃ was infused in the absence of RAP, 84.6% of the radioactivity was still present in the circulation after 60 min of perfusion. Taken together, these findings indicated that megalin retrieves DBP and 25-(OH) vitamin D₃/DBP complexes from the glomerular filtrate.

Prompted by the efficient megalin-mediated uptake of 25-(OH) vitamin D₃/DBP complexes, we analyzed whether this pathway also provided 25-(OH) vitamin D₃ for hydroxylation. To test for conversion, ³H-labeled vitamin D₃ metabolites were recovered from blood and urine of perfused animals (as in Table 2) and analyzed by high pressure liquid chromatography (HPLC). To distinguish between precursors taken up from the glomerular filtrate (luminal) or the circulation (basolateral), we used rats that had been perfused with ³H-25-(OH) D₃/DBP in the presence or absence of RAP for 30 to 40 min. At this time point, approximately 40% of ³H-25-(OH) vitamin D₃ has been filtered and reabsorbed by the proximal tubules. In the presence of RAP, the filtered precursor molecules were lost in the urine and only nonfiltered metabolites were available for conversion (Table 2). As depicted in Figure 5B, the vitamin D₃ metabolites recovered from the circulation of animals infused with tracer only were present as ³H-25-(OH) vitamin D₃ as well as ³H-1,25 (OH)₂ vitamin D₃. In contrast, only ³H-25-(OH) vitamin D₃, and no conversion products, were identified in plasma or urine of animals coinjected with RAP (Figure 5C). These findings demonstrate that filtered 25-(OH) vitamin D₃ molecules are hydroxylated and that inactivation of megalin prevents delivery of the precursor to tubular cells.

Vitamin D Deficiency and Bone Formation Defects in Megalin^{-/-} Mice

Megalin knockout mice excrete massive amounts of DBP in the urine (Figure 2). Because 1% of DBP molecules in the plasma are found in complex with 25-(OH) vitamin D₃, we reasoned that these animals also lose significant amounts of the vitamin. We therefore investigated the consequence of megalin deficiency for vitamin D homeostasis. Western blot analysis demonstrated that DBP levels in the circulation were similar in wild-type and in megalin^{-/-} mice, demonstrating that the plasma half-life of the protein was unchanged regardless if it was turned over by tubular degradation in wild-type mice or lost in the urine of knockout mice (Figure 6). However, urinary excretion of DBP in receptor-deficient animals resulted in the concomitant excretion of 25-(OH) vitamin D₃, which was not present in control urine (Figure 6). It was calculated that the mice lose 0.3–0.4 mg DBP and 4–5 ng 25-(OH) vitamin D₃ every 24 hr (see Experimental Procedures for details). Most importantly, urinary loss of the vitamin coincided with an 80% reduction in plasma 25-(OH) vitamin D₃ levels (Figure 6). Because plasma levels of 25-(OH) vitamin D₃ are an indicator of bioavailability of the vitamin, this suggested a status of vitamin D deficiency in megalin^{-/-} animals. Similar to patients and animal models with vitamin D deficiencies, megalin^{-/-} mice were characterized by severe bone formation defects. The mice were significantly retarded in growth (Figures 1A and 1B) and exhibited a dramatic reduction in density of the bones, as shown by contact X-ray imaging (Figures 7A and 7B). Histological evaluation of the vertebral bodies revealed highly irregular and scalloped bone surfaces compared to control animals (Figures 7C and 7D). The lack of continuity of the bone surfaces in the knockout mice was most likely a consequence of perforating osteoclastic resorptions occurring in high bone turnover conditions. Finally, disturbances in bone metabolism were confirmed by a 3.5-fold elevation in mean serum alkaline phosphatase levels (352 vs 106 U/l) and by increased concentrations of hydroxyproline in the urine (417 vs 193 μmol/l) as compared to control mice.

Discussion

Our findings demonstrate that complexes of 25-(OH) vitamin D₃ and DBP are filtered through the glomerulus

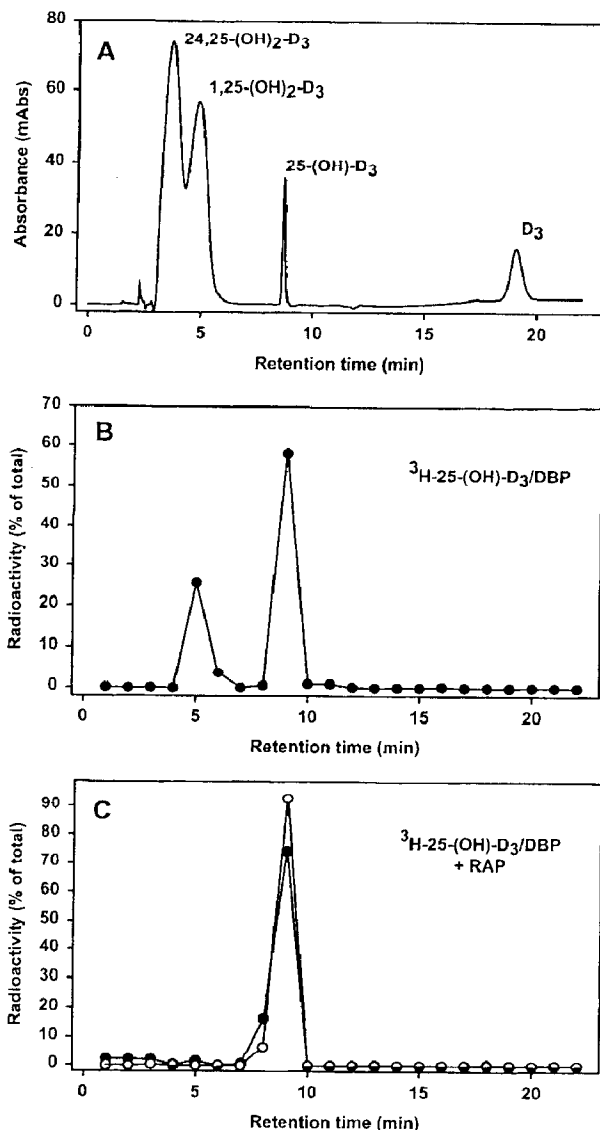


Figure 5. HPLC Analysis of Vitamin D₃ Metabolites in Rat Plasma and Urine

Vitamin D₃ metabolites were purified from plasma and urine samples of rats infused with ³H-25-(OH) D₃/DBP (B) or ³H-25-(OH) D₃/DBP + RAP (C) and subjected to high pressure liquid chromatography (HPLC) analysis on C₁₈ reverse-phased column (see Experimental Procedures). Radioactivity recovered in each fraction was expressed as the percentage of total radioactivity recovered (33,000 cpm in [B], 40,000–48,000 cpm in [C]). Representative profiles of individual animals of each group (six animals per group) are shown. The percent of radioactivity in the 1,25-(OH)₂ vitamin D₃ peak (B) varied between 10% (30 min perfusion) and 25% (45 min perfusion). Closed circles indicate blood; open circles indicate urine samples. For comparison, the HPLC profile of standard vitamin D₃ metabolites is shown in (A). D₃, vitamin D₃.

and reabsorbed in the proximal tubules by the endocytic receptor megalin. 25-(OH) vitamin D₃ molecules taken up via this receptor pathway are converted into 1,25-(OH)₂ vitamin D₃. Malabsorption of 25-(OH) vitamin D₃/DBP complexes in megalin knockout mice results in severe vitamin D deficiency and bone formation defects.

Previously, the role of DBP in the delivery of the 25-(OH) vitamin D₃ to the kidney was unclear. In established

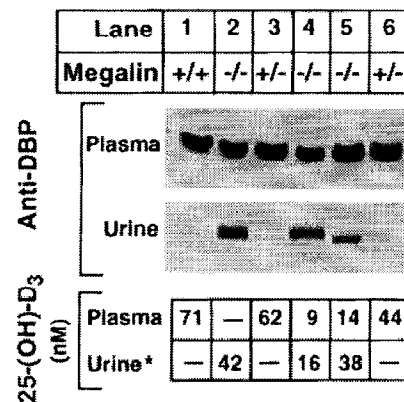


Figure 6. Quantification of DBP and 25-(OH) Vitamin D₃ in Mouse Plasma and Urine

Fifteen microliters of urine and 0.5 μ l of plasma obtained from mice of the indicated megalin genotypes were subjected to 10% nonreducing SDS-PAGE and immunoblot analysis using anti-DBP antiserum and ECL. 25-(OH) vitamin D₃ was measured in parallel plasma and urine samples as described in Experimental Procedures. Where no number is given, the measurement was below the detection limit of the assay (<5 nM). *, urine values were obtained on samples concentrated 30-fold by freeze-drying.

proximal tubular cell lines, uptake of 25-(OH) vitamin D₃ was observed when the steroid was added in free form, while little uptake was seen with complexes of 25-(OH) vitamin D₃/DBP (Keenan and Holmes, 1991). Therefore, it was hypothesized that in vivo uptake of the precursor also proceeds by diffusion of the free steroid through the plasma membrane. DBP was believed to solely regulate the amount of free 25-(OH) vitamin D₃ available in the circulation (Mendel, 1989; Haddad, 1995). This hypothesis was confounded by the observation that only 0.003% of 25-(OH) vitamin D₃ in the circulation is present in the free form (0.6 pg/ml plasma), whereas the rest is bound to DBP or serum albumin (20 ng/ml plasma) (Bikle et al., 1986). At the same time, approximately 0.3 to 0.5 ng of 25-(OH) vitamin D₃ are converted into 1, 25-(OH)₂ vitamin D₃ by the kidney every day (Bikle et al., 1985). The requirement for such massive intake of precursor strongly argues for additional steroid uptake pathways whereby the kidney gains access to the pool of bound 25-(OH) vitamin D₃. Based on our findings, it is likely that megalin-mediated endocytosis constitutes the pathway for delivery of the complexed precursor. Megalin knockout mice lose approximately 4–5 ng 25-(OH) vitamin D₃ in the urine over 24 hr (based on data in Figure 6). In wild-type animals, the same amount of 25-(OH) vitamin D₃ is taken up into epithelial cells via this receptor pathway, corresponding to ten times the amount of precursor metabolized per day. Besides significant efficiency, megalin-mediated uptake would also ensure cell type specificity for the delivery of the precursor. Because established proximal tubular cell lines (e.g., LLC-PK1) express very little megalin (E. I. C. et al., unpublished observations), this receptor pathway may have been missed in previous studies.

When megalin activity was blocked by RAP, no conversion products were recovered from rats infused with ³H-25-(OH) vitamin D₃ (Figure 5C). This result indicated

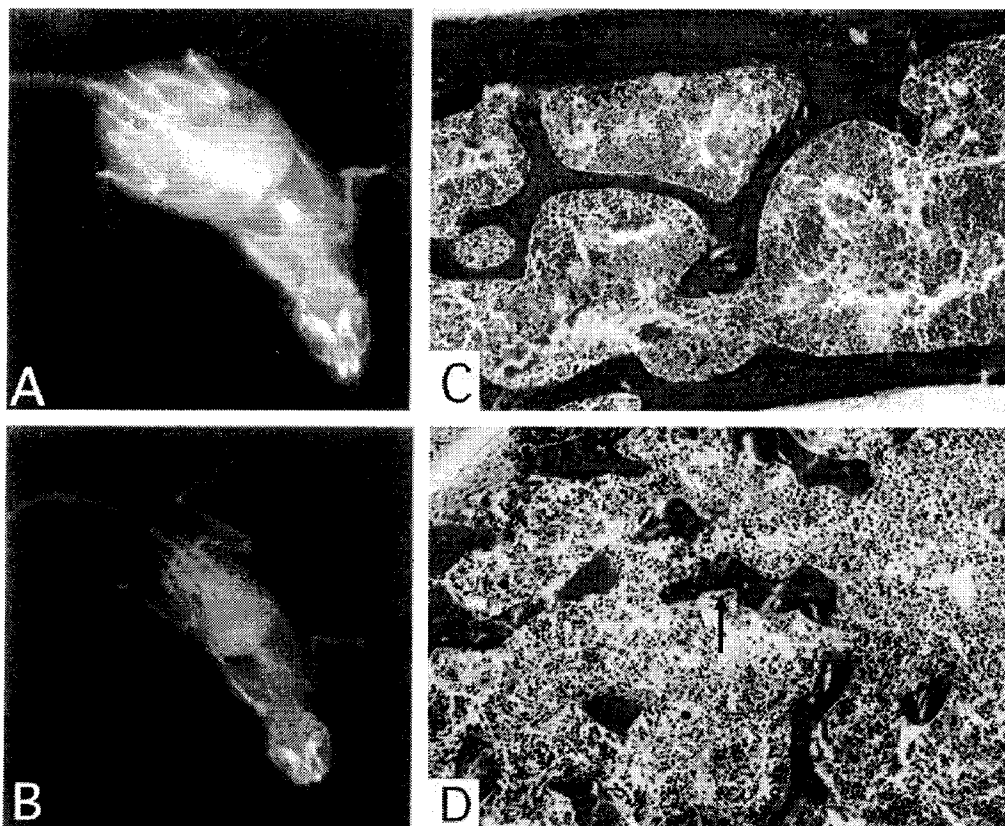


Figure 7. Bone Structure of Wild-Type and Megalin^{-/-} Mice

(A and B) Radiographic imaging of wild-type (A) and megalin^{-/-} littermates at 6 weeks of age (B).

(C and D) Histological analysis of the vertebral bodies of wild-type (C) and megalin^{-/-} mice (D). Undecalcified Goldner-stained sections were produced from the vertebral bodies. The arrow denotes irregular bone surfaces due to osteoclastic resorptions in the megalin-deficient mouse (D) compared to smooth connected cancellous bone surfaces in the control animal (C).

that receptor-mediated uptake from the lumen of the proximal tubule delivers precursors for hydroxylation. Such a role of tubular resorption processes in vitamin D metabolism is supported by studies in patients with renal defects. Similar to megalin knockout mice, patients that suffer from tubular resorption deficiency (Fanconi syndrome) exhibit vitamin D deficiency and bone disease (rickets, osteomalacia) (Harrison, 1953). Fanconi syndrome is caused by various genetic as well as environmental factors (e.g., heavy metal poisoning) and is characterized by an inability of the proximal tubules to reabsorb filtered macromolecules. One of the proteins excreted in the urine of Fanconi patients is DBP (Teranishi et al., 1983; Figure 3 inset). Therefore, the disturbances in calcium and bone metabolism in these patients might be explained in part by the urinary loss of 25-(OH) vitamin D₃ bound to the carrier. In addition, St. John et al. (1992) showed that in individuals with various degrees of renal failure, the glomerular filtration rate was directly correlated with the plasma concentrations of 1,25-(OH)₂ vitamin D₃. This finding suggests that glomerular filtration is a prerequisite for the generation of 1,25-(OH)₂ vitamin D₃.

Our results demonstrate that megalin constitutes a highly efficient pathway for delivery of 25-(OH) vitamin D₃ to tubular epithelial cells. These findings do not exclude other pathways for delivery of the precursor, such as

uptake of the free steroid from the circulation. The limited number (n = 8) and the small size of megalin^{-/-} animals precluded extensive characterization of electrolyte and endocrine parameters [e.g., parathyroid hormone and 1, 25-(OH)₂ vitamin D₃] to answer this question. Nevertheless, phenotypic analysis of mice genetically deficient for DBP strongly supports a role of megalin and its ligand DBP in the renal vitamin D₃ metabolism. In DBP^{-/-} mice, the plasma half-life of 25-(OH) vitamin D₃ is markedly reduced and urinary loss of the metabolite increased. This imbalance results in low plasma levels of 25-(OH) vitamin D₃ and 1,25-(OH)₂ vitamin D₃ and, as a consequence, in defects of bone metabolism (Cooke et al., 1997).

The present study identifies a pathway for renal uptake and activation of 25-(OH) vitamin D₃ involving the endocytic receptor megalin. Such a role of tubular endocytic processes in vitamin D homeostasis has not been known previously. We propose a model in which megalin mediates the tubular uptake of the steroid-carrier complexes filtered through the glomerulus (Figure 8). The carrier DBP is degraded in lysosomes, while 25-(OH) vitamin D₃ is converted into 1,25-(OH)₂ vitamin D₃ and resecreted into the circulation. This pathway exhibits striking resemblance to the cellular uptake of LDL by the LDL receptor. Both receptors mediate the endocytosis of lipid-carrier complexes by binding the apoprotein

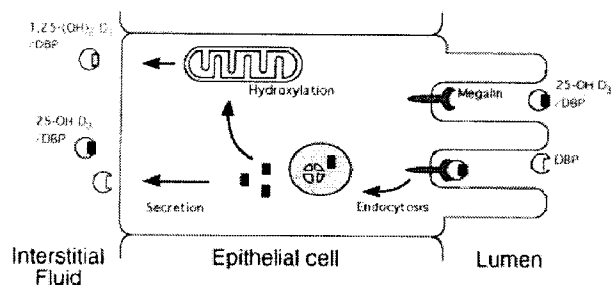


Figure 8. Model of Megalin Function in Renal Uptake and Activation of 25-(OH) Vitamin D₃

DBP and 25-(OH) vitamin D₃/DBP complexes are filtered through the glomerulus and endocytosed into the proximal tubular epithelium via megalin. The complexes are delivered to lysosomal compartments, where DBP is degraded and the vitamin released into the cytosol. 25-(OH) vitamin D₃ molecules are either secreted directly or hydroxylated to 1,25-(OH)₂ vitamin D₃ prior to release into the interstitial fluid. There, they are complexed by free DBP molecules.

moieties (apolipoprotein B-100 or DBP). The apoproteins are degraded while the steroids [cholesterol or 25-(OH) vitamin D₃] are delivered to the intracellular metabolism.

Similar to vitamin D metabolites, other steroid hormones are also transported in the circulation by specific plasma carrier proteins. These include sex hormone-binding globulin and corticosteroid-binding globulin. As suggested for DBP, these carriers are believed to keep steroids in a biologically inactive state and regulate the plasma concentration of free hormones that enter cells by diffusion (Siiteri and Simberg, 1986; Mendel, 1989). Such a concept requires nonspecific entry of steroids into all cells of an organism in order to find their intracellular hormone receptors. In contrast, receptor-mediated endocytosis of steroid-carrier complexes would offer the advantage of cell type-specific delivery. The existence of such uptake pathways for bound steroids was proposed because metabolic clearance rates were better correlated with the total than the free fraction of the hormones, indicating uptake of both free and bound steroids (Siiteri et al., 1982). Furthermore, steroid target tissues have been shown to express binding sites for carrier proteins (reviews in Siiteri and Simberg, 1986; Porto et al., 1995). Finally, endocytosis of testosterone-estradiol-binding globulin was demonstrated directly in the human breast cancer cell line MCF-7 (Porto et al., 1991). So far, the physiological significance of these endocytic pathways for steroid hormone action remained unclear. We now report one example for the biological importance of such endocytic pathways (megalin) inasmuch as they preserve plasma vitamin D levels and deliver to the kidney the precursor for generation of active 1,25-(OH)₂ vitamin D₃. Clearly, the resorption of filtered vitamin D metabolites in the renal proximal tubule is unique, as it requires especially efficient uptake pathways to prevent urinary loss of the vitamins. Nevertheless, it is intriguing to speculate that similar endocytic mechanisms may exist in other tissues. If confirmed for other hormonal systems, these findings not only have elucidated a central step in the vitamin D metabolism but may change current concepts in steroid hormone

metabolism reaching far beyond systemic calcium and bone metabolism.

Experimental Procedures

Materials and Sample Collection

Urine samples from patients with myeloma-associated Fanconi syndrome were kindly provided by P. Aucouturier (Hôpital de Paris), vitamin D₃ metabolites by A.-M. Kissmeyer (Leo Pharmaceutical, Copenhagen), anti-aquaporin IgG by S. Nielsen (University of Aarhus, Aarhus) and M. Knepper (NIH, Bethesda), anti-megalin antiserum by P. Verroust (Hôpital Tenon, Paris), and purified megalin by S. Moestrup (University of Aarhus, Aarhus). Anti-human DBP antiserum was purchased from DAKO (Hamburg), ³H-25-(OH) vitamin D₃ from Amersham (Braunschweig). Sterol/DBP complexes were prepared by incubating purified DBP with a 100-fold molar excess of ³H-25-(OH) vitamin D₃ for 16 hr at 4°C under N₂ in the dark. Mouse plasma was collected by retroorbital bleeding. For urine collection, mice were placed in metabolic cages for 16 hr and given 10% sucrose in drinking water. Urine samples obtained (approximately 8 ml/24 hr) were qualitatively indistinguishable from samples collected without sucrose load. Urine volume per hour and creatinine levels (~6 mg/dl) were identical in megalin^{-/-} and in control mice (not shown). Concentrations of DBP and 25-(OH) vitamin D₃ in plasma and urine were determined by semiquantitative Western blot analysis (for DBP) or competitive protein binding assay (for 25-(OH) vitamin D₃; Immunodiagnostik, Bensheim).

Receptor Binding Assay

Binding of DBP to megalin was quantified by BIAcore (Biosensor, Sweden) as described previously (Moestrup et al., 1998). A continuous flow of HBS buffer (10 mM HEPES, 3.4 mM EDTA, 150 mM NaCl, 0.005% surfactant P20 [pH 7.4]) passing over the sensor surface was maintained at 5 µl/min. The carboxylated dextran matrix of the sensor chip flow cell was activated by injection of 60 µl of a solution containing 0.2 M N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide and 0.05 M N-hydroxysuccinimide in water. Then, 180 µl of 10 mM sodium acetate (pH 4.5) containing 10 µg/ml purified rabbit megalin was injected. The remaining binding sites were blocked by subsequent injection of 35 µl 1 M ethanolamine (pH 8.5). The surface plasmon resonance signal from immobilized megalin generated 22,000 BIAcore response units (RU) equivalent to 34 fmol megalin/mm². To test DBP binding, rabbit megalin immobilized on CM5 sensor chip was incubated with 30 µg/ml human DBP in 10 mM HEPES, 150 mM NaCl, 1.5 mM CaCl₂, 1 mM EGTA (pH 7.4) or in 10 mM HEPES, 150 mM NaCl, 20 mM EDTA (pH 7.4), and the relative increase in response between megalin and control flow channels was determined. Kinetic parameters were determined by using the BIAevaluation 3.0 software.

Histological Analysis

The preparation of light and electron microscopical sections of kidney tissues has been described previously (Christensen et al., 1995). For immunolabeling, the sections were incubated with primary antibodies either at room temperature for 1 hr or overnight at 4°C, followed by incubation with peroxidase-conjugated secondary antibody and detection with diaminobenzidine. Binding of DBP and ¹²⁵I-RAP to kidney sections was carried out as published (Christensen et al., 1995). Briefly, the sections were incubated for 30 min with phosphate buffer containing 100 mM DBP or 1 µCi/ml ¹²⁵I-RAP. Bound DBP was detected by peroxidase-conjugated anti-DBP antibody and diaminobenzidine. For detection of ¹²⁵I-RAP, electron micrographs were subjected to autoradiography (Christensen et al., 1995).

Kidney Uptake Experiments

Microinfusion assays of proximal convoluted tubules of anesthetized Wistar rats have been described previously (Moestrup et al., 1995, 1996). A total of 0.5–2.7 pmol ¹²⁵I-DBP (in 50 nl) were infused into individual proximal tubules for 2 min in the presence or absence of 0.4 mg/ml RAP. Subsequently, urine was collected from a urethral catheter for 30 min. Tubular uptake was calculated as the difference

between trichloroacetic acid-precipitable counts microinfused versus recovered in urine. Perfusion of total rat kidneys was performed as follows: male Lewis rats fed a diet low in vitamin D and calcium (EF R/M 10 mm, Sniff, Soest) for 6 weeks were anesthetized and infused with 1% bovine serum albumin in 0.9% NaCl through the external jugular vein to enhance diuresis. The right kidney was clamped, and the aorta was occluded proximal and distal to the left renal artery. Subsequently, the left kidney was perfused for 10 min through a catheter placed in the aorta between the two occlusion sites with 1 ml of saline containing 50 pM ¹²⁵I-DBP or 12 ng/ml ³H-25-(OH) vitamin D₃ complexed with DBP. Where indicated, the infusion also contained 1 mg/ml RAP. Under these conditions, the tracer was allowed one pass through the glomerulus (10 min perfusion time). For extended filtration, the aorta was repaired and arterial blood flow through the kidney restored for 30 or 60 min by opening the proximal occlusion site. Therefore, the entire arterial blood flow was directed through the kidney. During the perfusion, urine was collected from the left urether. At the end of the experiment, blood was collected from the renal vein effluent, and the total amount of radioactivity in urine, blood, and kidney was determined. On average, 60% to 80% of total radioactivity injected was recovered. To isolate tritium-labeled vitamin D₃ metabolites, the urine and blood samples were precipitated with 66% acetonitrile. The supernatant was diluted to 15% acetonitrile and applied to activated C18 cartridges (SePak, Waters, Milford). The cartridges were washed with 3 ml of 70% methanol and 10 ml H₂O. The vitamin metabolites were then eluted in 4 ml acetonitrile and dried under constant nitrogen flow. Subsequently, the samples were resuspended in 100 µl isopropanol and chromatographed on C₁₈ reverse-phased column (VYDAC, #218TP104) in 85% methanol, 15% H₂O as published (Omdahl et al., 1980). Radioactivity was measured in all fractions and expressed as the percentage of total radioactivity recovered. For comparison, the retention profiles of unlabeled and tritium-labeled vitamin D₃ metabolites were determined.

Acknowledgments

We thank Linda Jacobsen for generating the DBP peptide sequence; Roger Bouillon and Hugo van Baelen for helpful discussions; and Christian Bönsch, Jørgen Gliemann, and David Russel for critical reading of the manuscript. The studies were funded by grants from the Deutsche Forschungsgemeinschaft, NIH (HL 20948), the Lysgaard, Carlsberg, and Novo Nordisk Foundations, the Danish Heart Association, and the Danish Medical Research Council.

Received September 18, 1998; revised December 15, 1998.

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The endocytic receptor megalin binds the iron transporting neutrophil-gelatinase-associated lipocalin with high affinity and mediates its cellular uptake

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Received 17 November 2004; revised 1 December 2004; accepted 1 December 2004

Available online 24 December 2004

Edited by Lukas Huber

Abstract Neutrophil-gelatinase-associated lipocalin (NGAL) is a prominent protein of specific granules of human neutrophils also synthesized by epithelial cells during inflammation. NGAL binds bacterial siderophores preventing bacteria from retrieving iron from this source. Also, NGAL may be important in delivering iron to cells during formation of the tubular epithelial cells of the primordial kidney. No cellular receptor for NGAL has been described.

We show here that megalin, a member of the low-density lipoprotein receptor family expressed in polarized epithelia, binds NGAL with high affinity, as shown by surface plasmon resonance analysis. Furthermore, a rat yolk sac cell line known to express high levels of megalin, endocytosed NGAL by a mechanism completely blocked by an antibody against megalin.

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Keywords: NGAL; Lipocalin; Megalin; Siderophore; Iron

1. Introduction

Lipocalins constitute a family of more than 30 proteins, found in species as separate as insects and man [1,2]. Lipocalins share a common structural fold, often in the absence of significant sequence homology, that forms a ligand-binding site (calyx) that is typically lined with hydrophobic residues [3,4]. Lipocalins function in general as transport proteins, e.g., odorant-binding protein, bilin-binding protein, and retinol-binding protein, but the physiological ligands have not been identified for all lipocalins. Lipocalins are extracellular proteins but some are involved in transport of substances that must be delivered intracellularly to exert a function, e.g., retinol. Very little is known about the receptors that mediate cel-

lular uptake of lipocalins and their ligands. It is not known whether the ligands are presented by the lipocalins to ligand receptors on cells or whether the lipocalins are taken up by receptors and then deliver their cargo intracellularly [5]. It is assumed that most lipocalins are taken up by membrane receptors but only two have been identified so far. One is a novel 51 kDa protein which binds tear-lipocalin (lipocalin-1 (Lcn-1)) and consequently is known as Lcn-1-Interacting Membrane Protein [6,7]. The other receptor is megalin [8], a multi-ligand endocytosis receptor that is expressed on a variety of epithelia, primarily such that have a high absorptive capacity such as tubular epithelial cells of kidneys, ileum, choroid plexus, and yolk sac [9]. Megalin belongs to the low density lipoprotein receptor family [8]. Megalin has been shown to bind the mouse lipocalins retinol-binding protein, α_1 -microglobulin, mouse major urinary protein, and odorant-binding protein [5,10].

We discovered a major human lipocalin, neutrophil-gelatinase-associated lipocalin (NGAL) (also known as lipocalin-2 (Lcn-2) or siderocalin), and have characterized its tissue expression [11]. NGAL is constitutively expressed in human neutrophils but is also induced in epithelial cells when these are engaged in inflammation [12–14]. This tissue expression indicates that NGAL is involved in innate immunity. This notion was supported when siderophores were identified as NGAL ligands [15] and followed by the recent demonstration of decreased survival of mice with a targeted disruption of the NGAL (Lcn-2) after intraperitoneal challenge with *Escherichia coli* [16]. Siderophores are extremely strong iron chelators secreted by microorganisms when iron is limiting [17]. Siderophores can extract iron out of iron-binding proteins such as transferrin and lactoferrin for subsequent uptake by specific siderophore receptors on the microorganisms. In this way, microorganisms can secure a supply of this essential nutrient at the expense of their host [17]. It has been shown that apo-NGAL is able to prevent siderophore producing *E. coli* from dividing and that this restrain on bacterial growth is alleviated by supplying iron in excess of the iron-siderophore binding capacity of NGAL [15]. However, such antimicrobial activity can be expected to be temporary because several microorganisms secrete proteases that may degrade NGAL even though NGAL is known to be a very protease resistant molecule [18]. NGAL would therefore be expected to have a more sustained antimicrobial effect, if NGAL is taken up by host cells.

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Abbreviations: BN, Brown Norway; Lcn-1, lipocalin-1; Lcn-2, lipocalin-2; LRP, LDL-receptor related protein; NGAL, neutrophil-gelatinase-associated lipocalin; RAP, receptor associated protein; SPR, surface plasmon resonance

This has been further indicated by the recent observation that the mouse ortholog of NGAL, 24p3, is expressed in the fetal kidney and delivers iron to cells at the stage where differentiation of the budding kidney epithelia takes place during organogenesis of the kidneys [19,20]. The rapid clearance of NGAL from the circulation indicates a mechanism of cellular uptake [21]. In addition, NGAL has been inferred as a factor inducing apoptosis through a receptor mediated mechanism in IL-3 dependent cells such as bone marrow cells in the mouse [22], but this has not been confirmed [23].

Since none has yet been identified, we decided to test whether megalin could also function as a receptor for NGAL.

2. Materials and methods

Recombinant human NGAL was generated either as apo-NGAL, i.e., without bacterial siderophore, in Sf9 insect cells using the Baculovirus technique as previously described [24], or as NGAL with *E. coli* siderophore as detailed in [15,25], with the modification that 50 ml overnight culture of *E. coli* was added to 500 ml LB medium and incubated for 90 min. Then, 0.1 mM IPTG and 0.1 mg/ml desferoxamine (Desferal, Roche) were added to induce bacterial growth (IPTG) and secure iron deprivation and enterobactin production by the bacteria. After 3 h, 0.1 mg/ml FeCl_3 was added and the bacteria incubated for another 90 min. The bacteria were then pelleted by centrifugation and formed a bright red pellet. The bacteria were lysed and rhNGAL was isolated as described [25]. The isolated NGAL was scanned as described from 250 to 800 nm, and showed the characteristic peaks at 330 and 500 nm indicative of enterobactin- Fe^{3+} complex in the lipocalin pocket [15]. These were absent in the apo-NGAL from Sf9 cells (Fig. 1). It was ascertained that desferoxamine did not bind to apo-NGAL by incubating apo-NGAL with desferoxamine and FeCl_3 .

Mouse monoclonal anti-NGAL antibody was used for Western blotting [26]. NGAL was quantitated as described by Kjeldsen et al. [26].

Megalin was purified by receptor-associated protein (RAP) affinity chromatography from human kidney cortex according to standard procedures [27]. Human LDL-receptor related protein (LRP) was purified by α_2 macroglobulin-affinity chromatography as described [28]. Purified sheep polyclonal antibodies against rat megalin, previously described [29], were used to block uptake of NGAL by megalin. Purified sheep non-immune IgG served as negative control.

3. Surface plasmon resonance analysis

The binding to megalin and LRP was studied by surface plasmon resonance (SPR) analysis on a Biacore 2000 instrument (Biacore, Sweden). The procedure was as follows: Biacore sensor chips type CM5 were activated with a 1:1

mixture of 0.2 M *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccinimide in water according to the manufacturer's instructions. Megalin and LRP were immobilized at a concentration of 10 $\mu\text{g/ml}$ in 10 mM sodium acetate, pH 4.5, and the remaining binding sites were blocked with 1 M ethanolamine, pH 8.5. A control flow cell was made by performing the activation and blocking procedures only. The resulting receptor densities were 25–30 fmol receptor/ mm^2 . Samples were dissolved in 10 mM HEPES, 150 mM NaCl, 1.5 mM CaCl_2 , 1.0 mM EGTA, and 0.005% Tween 20, pH 7.4, or in 10 mM HEPES, 150 mM NaCl, 20 mM EGTA, and 0.005% Tween 20, pH 7.4. Sample and running buffer were identical. Regeneration of the sensor chip after each analysis cycle was performed with 1.6 M glycine-HCl buffer, pH 3.0. The Biacore response is expressed in relative response units (RU), i.e., the difference in response between protein and control flow channel (an activated but uncoupled flow cell). Kinetic parameters were determined by BIAevaluation 4.1 software using a Langmuir 1:1 binding model and simultaneous fitting of all curves in the concentration range considered (global fitting).

4. Cellular uptake of NGAL

Megalin-expressing Brown Norway (BN) rat yolk sac epithelial cells transformed with mouse sarcoma virus (BN cells) [30] were grown on two-chamber Permanox slides (Nunc, Roskilde, Denmark) and washed in PBS, pH 7.4. Cells were then incubated for 1 h at 37 °C in HyQ-CCM5 serum-free medium (Hyclone, Logan, Utah) containing 1% BSA (w/v) and $\approx 60 \mu\text{g/ml}$ rhNGAL labeled with Alexa-488 (Molecular Probes, Leiden, The Netherlands). To some cells was also added sheep polyclonal anti-rat megalin IgG antibody (200 $\mu\text{g/ml}$) or sheep non-immune IgG antibody (200 $\mu\text{g/ml}$). After incubation, cells were washed in PBS, pH 7.4, and fixed in 4% formaldehyde for 1 h at 4 °C. Subsequently, cells were washed in PBS, pH 7.4, containing 0.05% Triton X-100 and incubated with rabbit anti-rat cubilin antibody [31] (10 $\mu\text{g/ml}$) in this buffer for 1 h at room temperature in order to visualize the megalin-expressing structures of the cells. After washing in PBS, 0.05% Triton X-100, cells were incubated for 1 h at room temperature with Alexa-594-conjugated secondary anti-rabbit IgG (Molecular Probes) diluted 1:200 in the same buffer. Slides were washed in PBS, pH 7.4, mounted in Dako fluorescent mounting medium (DakoCytomation, Glostrup, Denmark) and analyzed

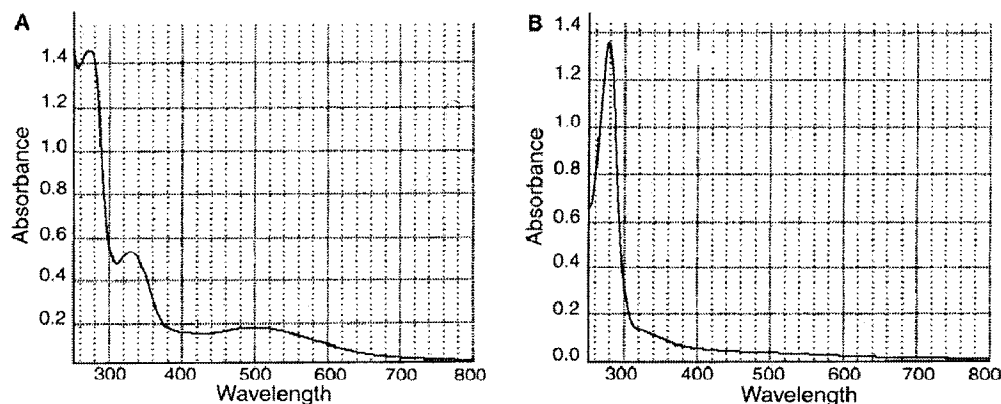


Fig. 1. Absorption spectrum of NGAL. NGAL (1.2 mg/ml) with siderophore (A) and without siderophore (B) in PBS was scanned against PBS.

using a Zeiss LSM-510 confocal microscope (Zeiss, Jena, Germany).

5. Results

To test the hypothesis that NGAL might bind to megalin, studies were conducted using SPR technique with matrix bound megalin. Fig. 2 shows that binding ($K_d = \sim 60$ nM) of apo-NGAL to megalin occurs with high affinity. Similar affinity was measured with siderophore-bound NGAL (not shown). The NGAL binding was prevented by EDTA, indicating that the LDL-receptor type-A repeats of megalin are involved in the binding of NGAL. Since this motif is also found in the LRP [32], we tested the binding of NGAL to LRP. However, only a very weak signal was recorded (not shown) indicating that this receptor has no major role in NGAL clearance.

The role of megalin in cellular uptake of NGAL was further investigated in a rat yolk sac cell line that is known to express high amounts of megalin [30]. Fig. 3 shows that endocytosis of fluorescently labeled NGAL by this cell line was extensive as seen by the appearance of intracellular NGAL (Alexa-488, green fluorescence). A sheep polyclonal anti-megalin antibody completely prevented cellular uptake of NGAL by these cells (Fig. 3B), thus indicating that megalin is involved in mediating the cellular uptake of NGAL by the cells. Cubilin, a receptor colocalizing with megalin and recycling between coated pits and endosomes, was visualized with Alexa-594 coupled antibody (red fluorescence). The different localization of NGAL and cubilin indicates that internalized NGAL is not recycled but segregated from the receptor and targeted to endosomes and lysosomes.

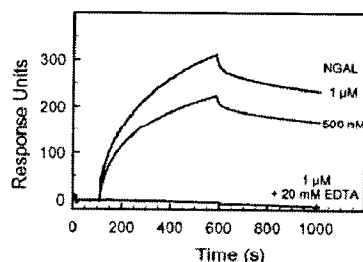


Fig. 2. Surface plasmon resonance analysis of the binding of NGAL to purified megalin. Binding of 500 nM and 1 μ M NGAL to megalin. No binding of NGAL was seen in the presence of calcium-complexing EDTA.

6. Discussion

Neutrophil-gelatinase-associated lipocalin appears to be a protein of importance in iron metabolism both during organogenesis [19,20] and in host defense [15,16], and possibly also in tumorigenesis [33,34]. The ability of NGAL to sequester iron and thus prevent its utilization by growing microorganisms has been demonstrated in vitro [15], and NGAL/24p3 has been shown to mediate uptake of iron by the developing kidney [19]. Although not a priori apparent, it is highly likely that the ability of NGAL to prevent iron utilization by microorganisms as demonstrated in vitro is ultimately dependent on cellular uptake of iron, since NGAL might be degraded by (microbial) proteases and the siderophore-bound iron retrieved by microorganisms if NGAL is not taken up by the host epithelial cells. Certainly, the essential role of NGAL in inducing epithelial cell differentiation in the developing kidney is dependent on cellular uptake and delivery of NGAL bound iron to the epithelial cells [19]. It is similarly likely – but entirely speculative – that the expression of NGAL by a variety of epithelial tumors endows these with an iron retrieving mechanism that adds to the growth potential of the tumors [33–35], and thus that for NGAL to exert its function, be it in host defense or during cellular growth and differentiation, a cellular receptor is needed which mediates uptake of NGAL with high affinity. We demonstrate here that megalin acts as such a cellular receptor for NGAL. Megalin is also known to bind another iron-binding protein expressed and secreted from human neutrophils, lactoferrin [36,37]. Although the ligands for megalin are very diverse, positively charged amino acids have been shown to be critical for binding of several ligands to this receptor [32]. The *pI* of NGAL of 8.4 distinguishes NGAL from most other lipocalins [4]. A number of positive charges can be identified on NGAL from analysis of its crystalline structure [15]. The affinity of megalin for NGAL as determined by plasmon resonance is much higher than that observed for other lipocalins and may likely relate to the unique positive charge of NGAL among lipocalins.

The tissue expression of megalin fits well with the induction of NGAL expression during inflammation. NGAL is highly expressed by type 2 pneumocytes during inflammation [12]. These also express megalin [8]. NGAL is also highly expressed by epithelial cells of the intestines during inflammation [13]. These also express megalin [9].

It is noteworthy that we were not able to show any selectivity of megalin for siderophore NGAL versus apo-NGAL. It may be argued that in this way apoNGAL may decrease the

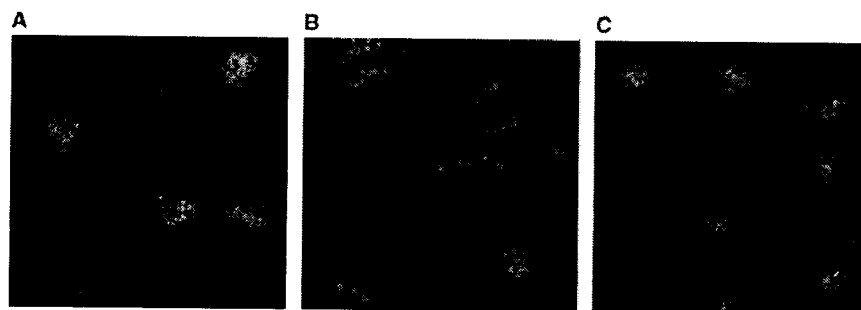


Fig. 3. Confocal fluorescence microscopy analysis of uptake of Alexa-488-labeled NGAL in BN cells. BN cells were incubated for 1 h with rhNGAL (green) in the absence (A) or in the presence (B) of anti-megalin IgG or non-immune IgG (C). The red color represents Alexa-594 staining for cubilin, a receptor colocalizing with megalin [38].

efficiency by which megalin takes up siderophore-NGAL – this would however only be a problem if the ability of megalin to bind and endocytose NGAL is a limiting factor. The studies of uptake indicate that the capacity of megalin to endocytose NGAL is very high.

Our studies do not exclude the possibility that other receptors other than megalin may be involved in cellular uptake of NGAL.

Acknowledgments: This study was supported by grants from the Danish Medical Research Council. The expert technical support of Charlotte Horn is greatly appreciated.

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